Computational Predictions of Drug Biodistribution and Drug Action in the Central Nervous System

BY

Ying Hsu
B.S. in Bioengineering from University of Illinois at Chicago

THESIS
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Defense committee:
Andreas Linninger Ph.D., Chair and advisor
Simon Alford Ph.D., Department of Biological Sciences
Sukhraaj Basati Ph.D., System Science
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Table of Contents

SUMMARY ................................................................................................................................. V

CHAPTER I. EFFECT OF SPINAL NERVES ON CEREBROSPINAL FLUID FLOW ........................................ 1
  ABSTRACT ............................................................................................................................... 1
  INTRODUCTION .................................................................................................................. 2
  METHODS .............................................................................................................................. 3
  RESULTS ................................................................................................................................. 6
  DISCUSSION .......................................................................................................................... 9
  CONCLUSION ......................................................................................................................... 10

CHAPTER II. EFFECT OF TRABECULAE ON CEREBRAL SPINAL FLUID FLOW AND DRUG MIXING .................. 11
  ABSTRACT ............................................................................................................................... 11
  METHODS .............................................................................................................................. 12
  RESULTS ................................................................................................................................. 15
  DISCUSSIONS ...................................................................................................................... 17
  CONCLUSION ....................................................................................................................... 18

CHAPTER III. GENERATION OF PATIENT-SPECIFIC COMPUTATIONAL MODELS. .............................................. 19
  ABSTRACT ............................................................................................................................... 19
  INTRODUCTION .................................................................................................................. 20
  METHODS .............................................................................................................................. 20
  RESULTS ................................................................................................................................. 21
  DISCUSSION .......................................................................................................................... 23
  CONCLUSION ....................................................................................................................... 23

CHAPTER IV. THE FREQUENCY AND MAGNITUDE OF CEREBROSPINAL FLUID PULSATIONS INFLUENCE INTRATHecal DRUG DISTRIBUTION - KEY FACTORS FOR INTERPATIENT VARIABILITY ................................................. 24
  ABSTRACT ............................................................................................................................... 24
  INTRODUCTION .................................................................................................................. 25
  METHODS .............................................................................................................................. 26
  RESULTS ................................................................................................................................. 29
  DISCUSSION .......................................................................................................................... 35
  CONCLUSIONS ....................................................................................................................... 38

CHAPTER V. THE DEVELOPMENT OF SINGLE-CELL KINETIC MODEL FOR GENE SILENCING THERAPIES .......... 39
  ABSTRACT ............................................................................................................................... 39
  METHODS .............................................................................................................................. 40
  RESULTS ................................................................................................................................. 42
  DISCUSSION .......................................................................................................................... 46
  CONCLUSION ....................................................................................................................... 47

CHAPTER VI. MEDICAL IMAGE-BASED SYSTEMATIC DESIGN OF HUMAN GENE SILENCING THERAPIES .......... 49
  ABSTRACT ............................................................................................................................... 49
  INTRODUCTION .................................................................................................................. 50
  METHODOLOGY ................................................................................................................. 51
  RESULTS AND DISCUSSION ............................................................................................... 53
  CONCLUSIONS ....................................................................................................................... 55

CHAPTER VII. ACTIVATION OF A NEUROPROTECTIVE TRANSCRIPTION FACTOR AND AQUAPORIN-4 UPREGULATION IN BRAIN ASTROCYTES .................................................................................... 56
  ABSTRACT ............................................................................................................................... 56
Summary.

Drug delivery to the central nervous system (CNS) is widely used for the administration of spinal anesthetics, chemotherapeutics, medications for pain management, and spasticity. Many novel applications are being developed with an accelerated pace, including the delivery of short interfering RNA (siRNA), nerve growth factors for traumatic spinal cord injury, and viral vectors. Despite its wide applications, severe side effects occur even in well-tested clinical applications, mostly due to patient-to-patient variability in drug distribution and metabolism. The optimal design of infusion therapies is of great importance for the translation of bench top results to clinical therapies.

In the past, this translation is mainly accomplished by animal testing. Even after trial and error testing on rodents and primates, the accurate scaling of dosage is still required to achieve similar distribution and effect in humans. Developing infusion therapies based solely on animal testing has some disadvantages. 1) There is a difference between animal and human CNS physiology. 2) Incorrect scaling leads to ineffective dosing or overdose. 3) trial and error animal experiments are cost-intensive. 4) Animal experiments do not reflect patient-to-patient differences in anatomy, drug biotransport, and metabolic rates, and they cannot address the important issue of subject-specific infusion therapies.

Despite the advances in medical imaging technologies, mathematical modeling, pharmacokinetics, and biochemistry, we still cannot predict the fate of drugs and their biodistribution after an infusion of a novel drug. Therefore, even though infusion guidelines exist for well-tested pharmaceuticals, there is not a reliable means to predict drug distribution and effect a priori for a novel therapy. This is due to an incomplete understanding of drug transport and metabolic mechanisms in the CNS. The prediction of dose-response for a novel application for a specific subject is, in fact, possible.

After infusion, drug molecules disperse in the spinal canal due to the pulsating motion of the cerebrospinal fluid (CSF). This pulsating motion has been measured by magnetic resonance imaging (MRI), and its origin is the expansions of the cerebral vasculature inside the cranium at every heart beat. Even though the pulsatile phenomenon of CSF in the spine is well known, no studies exist that investigate the pulsatile dynamics of CSF and its relationship to drug distribution. In this thesis, the pulsations of CSF in the spine are quantified with
CINE-MRI, and the effect of physiological pulsatile motion on drug distribution speed was quantified using single photon emission computed tomography (SPECT) on a spine model.

Drug distribution in the spine is also a function of spine anatomy. The spinal canal is filled with anatomical fine structures that act as resistance to fluid flow and drug transport such as nerves, filamentous trabeculae, septa, and ligaments. Predictions of drug transport and action at the target site require an understanding of these mechanisms. The advances in medical imaging technologies have opened the door for a new predictive tool for designing drug infusion therapies - patients-specific computational design. The application of this technique to the CNS is still in the budding stage. The uptake and biochemical reactions of the drug are prerequisites for a drug to exert an effect in an organ or tissue. Some drugs have simpler mechanisms such as the binding to target receptors, while other drugs have more complex action mechanisms including endocytosis, interaction with the transcriptional or translational machinery, or interference with protein synthesis. To predict drug effect in a complex organism, the biochemical reactions of the drug must be taken into account besides from its biodistribution in fluid and tissue. For this reason, the computations of deterministic and stochastic biochemical reactions, mathematical assembly of gene transcriptional and translational cascades, and finally the integration of patient-specific models with drug pharmacokinetics will be covered in this thesis as well.

In summary, the generation of patient-specific models from medical imaging, the application of computational fluid dynamics to simulate CSF pulsations, the prediction of drug biodistribution after infusion, and finally the prediction of drug action at the target site will be introduced in the chapters of this thesis.
Chapter I. Effect of Spinal Nerves on Cerebrospinal Fluid Flow

Abstract

Background. The spinal canal is not a smooth cavity for cerebrospinal fluid flow and infused drugs. Fine anatomical structures such as nerves and arachnoid trabeculae populate the spinal canal cavity, acting as obstruction for fluid flow and species transport. The human spine contains 31 pairs of nerves that are connected to the spinal cord. This study investigates on how the existence of protruding nerves inside the spinal canal will influence the fluid flow across the spinal canal.

Methodology. Computational fluid dynamic analysis is carried out to investigate the effect of nerves on cerebrospinal fluid flow. Two computational models resembling the human spinal canal, one with spinal nerves and one without nerves, are constructed according to physiological dimensions. Computational fluid dynamics are performed to derive fluid flow patterns and pressure drop characteristics inside these domains. A sinusoidal wave function was used to approximate the pulsatile CSF motion inside the spinal canal.

Results. Computations showed that spinal nerves added 29% of pressure drop during a constant flow compared to a control model without nerves. On the other hand, for a pulsatile flow they added a 10% pressure drop. Results showed that spinal nerves significantly increased fluid flow resistance in the spine.

Conclusions. For future studies of CSF flow patterns inside the human central nervous system, the construction of spinal nerves will significantly impact the prediction of flow fields.
Introduction

Intrathecal delivery is a commonly used infusion method clinically. This method has tremendous potential in the clinical applications in pain management or anesthesia. The optimal dosing and delivery module of intrathecal therapeutics will benefit tremendously from the construction of computational models that can accurately predict spinal fluid dynamics and drug distribution.

Drug distribution in the spine is a function of pulsatile cerebrospinal fluid (CSF) flow, infusion parameters, as well as the anatomical structure of the spine. Prior research has indicated that the pulsation of the CSF is one of the most important reasons for the fast distribution speed of intrathecal drugs. CSF pulsations in the subarachnoid space is primarily caused by the expansions of cerebral vasculature at every heart beat. Pulsations of CSF within a complex spatial geometry creates a flow pattern that may have characteristics of turbulent or mixed flow. The spinal canal is filled with microstructures such as septa, arachnoid trabeculae, and pairs of nerves connected to the cord. In terms of drug delivery, the presence of these microstructures may (1) provide additional resistance to CSF flow, and (2) increase the mixing speed of injected drugs by introducing micro-eddies and disrupting laminar flow.

The septa and trabeculae are structures in the micron scale, and are not always visible on medical images. Figure 1 shows a scanning electron micrograph of the lumbar spinal cord of a 15-month-old child as imaged by Nicholas et al., where septa, leptomeningeal, and the dentate ligaments are visible. However, these structures may disrupt laminar flow, creating micro-eddies inside the canal.

Figure 1. Scanning electron micrograph of the lumbar spinal cord of a 15-month-old child measured by Nicholas et al. In the figure: (IL) leptomeningeal layer, (S) septa, (A) arachnoid layer, (D) dentate ligaments.
Computational studies done by Stockman has predicted that small obstructions like nerves and trabeculae have minor impact on the bulk fluid flow, but has great impact on mixing and dispersion of tracer across the subarachnoid space using the Lattice Boltzmann method. However, this issue has never been investigated using continuum mechanics. In this chapter, the effect of spinal nerves inside the spinal canal is investigated using computational fluid dynamics (CFD).

Methods

Computational Models

Three dimensional spine models were created using the software GAMBIT. Two shorter models of 7.5cm in length were built, one with nerves and the other served as a control. The short control model (sControl) is the control model that has no spinal nerves, and seven pairs of spinal nerves were constructed throughout the length of the short nerve model (sNerve). The long control model (lControl) and long nerve model (lNerve) have similar properties with lengths of 22.5cm. The inner and outer diameters of the models as well as the size of nerve roots were chosen to match human dimensions, see Table 1. The models are presented in Figure 3. The lControl and lNerve models contain injection sites with radius of 0.03 cm in the middle of the outer wall of the canal for studying species transport. However, the injection of species was not performed due to the fact that drugs rapidly leave the computational domains in pulsatile flow.

Table 1. Dimensions of spinal canal models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Cord diameter</th>
<th>Canal diameter</th>
<th>Nerve thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>10 mm</td>
<td>20 mm</td>
<td>2.25 mm</td>
</tr>
<tr>
<td>Literature</td>
<td>5.3-5.6 mm(6)</td>
<td>16-20 mm(9)</td>
<td>1.8-2 mm(10)</td>
</tr>
<tr>
<td></td>
<td>6-12 mm(7-8)</td>
<td>13-15 mm (Lee 1994)</td>
<td></td>
</tr>
</tbody>
</table>
The four geometries were discretized into computational meshes. Number of tetrahedral cells (round to nearest thousands) in each model is presented in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Short Control Model</th>
<th>Short Nerve Model</th>
<th>Long Control Model</th>
<th>Long Nerve Model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Tetrahedrons</strong></td>
<td>880,000</td>
<td>300,000</td>
<td>400,000</td>
<td>800,000</td>
</tr>
</tbody>
</table>

**Boundary Conditions.**

A complete list of boundary conditions is presented in Table 3.

*Simulations with constant flow CSF*

The effect of nerves on the pressure drop across the spinal canal while subject to constant (non-pulsatile) fluid flow was studied using sControl and sNerve models. A constant fluid velocity of 0.5 cm/sec was applied at the inlet boundary while the outlet is assigned a pressure boundary condition.
Simulations with pulsatile flow CSF

The effect of nerves on the pressure drop across the spinal canal while subject to pulsatile fluid flow was studied using sControl and sNerve models. The pulsation of the CSF is represented by the sinusoidal function shown in Equation 1 to resemble the pulsating effect inside the spinal canal. Pressure data was gathered along a longitudinal line within the spinal canal. The exact procedures used for FLUENT simulations were included in the appendix.

\[ V = 0.01 \sin (6.2318t) \]  
\[ \text{Equation 1} \]

Effect of spinal nerves on drug distribution across the spinal canal

For studying drug distribution, long models (lControl and lNerve) were used to allow larger dispersion distances for drug molecules. As a result, the drug is reduced significantly from one of the injection amount from Chambers et al, 1982. During the simulation, the drug solution is injected with speed of 0.000471 m/s into the two models through the opening. Instead of the sinusoidal waveform applied previously, a more complex waveform was used to described CSF motion.

Equation 2 describes the pulsating function of the CSF fluid with waveform of cardiac cycle.

\[ V = (-4.29 \cos(y) - 2.427 \sin(y) + 4.971 \cos(2y) - 0.05327 \sin(2y) + 0.4384 \cos(3y) - 2.526 \sin(3y) + 1.507 \cos(4y) + 0.9098 \sin(4y) - 1.03 \cos(5y) - 0.1822 \sin(5y))/100.0 \]  
\[ \text{Equation 2} \]

Table 3. Boundary conditions for computational simulations

<table>
<thead>
<tr>
<th>Boundary Name</th>
<th>Boundary Type</th>
<th>Boundary Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>Pressure drop study: wall</td>
<td>Pressure drop study: wall (zero diffusivity flux)</td>
</tr>
<tr>
<td></td>
<td>Drug distribution study: velocity inlet</td>
<td></td>
</tr>
<tr>
<td>Inlet</td>
<td>Velocity inlet</td>
<td>Pressure drop study: Sinusoidal function</td>
</tr>
<tr>
<td>Outlet</td>
<td>Pressure outlet</td>
<td>Gauge pressure=0 Pascal</td>
</tr>
<tr>
<td>Outer wall</td>
<td>Wall</td>
<td>Zero diffusivity flux</td>
</tr>
<tr>
<td>Inner wall</td>
<td>Wall</td>
<td>Zero diffusivity flux</td>
</tr>
</tbody>
</table>
Assumptions and Numerical solution

The bulk fluid is assumed to be a Newtonian fluid which is incompressible with constant viscosity. No slip boundary conditions were applied at the walls. The computations were performed in a commercial software, FLUENT. Equations for momentum balance, species transport, continuity, and pressure were presented in Equation 3 through Equation 5. A detailed mathematical decomposition of the numerical solution of the Navier-Stokes equation is described in Appendix 1. This report containing the numerical solution of Navier-Stokes equation in MATLAB was completed in collaboration with colleague Ian Gould from our laboratory.

\[
\frac{\partial}{\partial t} (\rho \vec{v}) + \nabla \cdot (\rho \vec{v} \vec{v}) = -\nabla p + \nabla \cdot (\tau) + \rho \vec{g} + \vec{F} \tag{Equation 3}
\]

\[
\frac{\partial C}{\partial t} + \vec{v} \nabla C = \nabla D \nabla C + R \tag{Equation 4}
\]

\[
\nabla \cdot \vec{v} = 0 \text{ (For incompressible flow)} \tag{Equation 5}
\]

These fundamental equations were solved in FLUENT with the finite volume method. The numerical algorithm applied in FLUENT was the SIMPLE (Semi-Implicit Method for Pressure-Linked equations) algorithm, and its programmatical implementations were described in a previous chapter.

Results

**Pressure drop across the spinal canal with a constant flow**

The pressure drop, indicating total resistance for fluid flow through this domain, for a constant flow was shown in Figure 4. At the outlet of the models, pressure was set to 0 pascal (blue). For the sControl model, the pressure developed at the inlet due to fluid flow was 0.23 Pascal. For the sNerve model, the pressure developed at the inlet was 0.32 Pascal. The result shows that spinal nerves increase the pressure drop across the spinal canal by 29% for a constant fluid flow.
Figure 4: Pressure drop shown on the spinal cord surface across the spinal canal for a constant fluid flow. The color scale shows pressure in pascals. Simulations showed that nerves increased fluid flow pressure drop by 29% in constant flow.

Figure 5 shows pressure drop in the sControl (yellow curve) and sNerve (blue curve) models along the longitudinal length of the models. The magenta curve was pressure drop near the spinal nerves in the sNerve model.

Figure 5. Pressure drop across the models for a constant flow of 5 mm/sec. The pressure drop across the model with nerves is 29% higher than that across control model. Pressure values derived from a line placed near the nerves shows that pressure increases near a nerve, indicating the rise in fluid flow resistance near these obstructions.
Pressure drop across the spinal canal with a pulsatile flow

A pulsatile flow with sinusoidal waveform was applied at the inlet boundaries while a fixed pressure of 0 pascal was applied at the outlet boundaries. For the sControl and sNerve models, the difference in pressure drop was 0.4 Pascal. Spinal nerve added an increase in resistance to pulsatile fluid flow of 9%.

Figure 6. Pressure drop across the sNerve and sControl with a pulsatile fluid flow. Pressure values derived from a line placed near the nerves shows that pressure increases near a nerve, indicating the rise in fluid flow resistance near these obstructions.

In a pulsatile flow field, the pressure field changes with time, indicating that the pressure drop across a model will not be the same throughout 1 pulsatile cycle. Therefore, pressure drop across the two models throughout one pulsatile cycle was quantified and shown in Figure 7.
As shown in Figure 7, pressure drop shows dynamic pulsations in both models. The maximum pressure drop in the sNerve model was higher than in sControl model. The average pressure drop across models were calculated to be 3.75 Pascal and 4.13 Pascal for sControl and sNerve models, respectively. The different in averaged pressure drop was 0.37891 Pascal, indicating that spinal nerves added 10% pressure drop and fluid flow resistance across the spinal canal.

Discussion.

The spinal canal is filled with fine structures that may act as obstacles for CSF flow. This computational investigation showed that spinal nerves increase the pressure drop across the spinal canal by 29 percent when subject to a constant fluid flow. However, CSF flow in the spinal canal does not have a constant velocity. Rather, the flow has a pulsatile motion caused by cerebral vascular expansions at the frequency of the heart rate. In the presence of pulsatile flow mimicking CSF motion inside the human spinal canal, nerves increased pressure drop by 10%. This could indicate that the presence of the spinal nerves inside the spinal canal account for a 10% increase in flow resistance.
The pressure values inside the spinal canal are of great interest to studying abnormal CSF flow patterns. This computational study demonstrated that the presence of nerves added about 10% of flow resistance, which is not negligible. Therefore, the reconstruction of a subject-specific computational model in subsequent studies will include the spinal nerves inside the spinal canal for an accurate depiction of the CSF flow.

Also, spinal canal wall and spinal nerves are elastic materials. Spinal canal is known to exhibit compliance especially at the lower spine. In the future, moving boundaries for simulating compliance of the spine will be necessary to capture realistic CSF flow within the spinal canal.

Conclusion

Spinal nerves inside the spinal canal added 29% of flow resistance for a constant flow, and added a 10% resistance during pulsatile flow, respectively. This study demonstrates that spinal nerves inside the spinal subarachnoid space significantly increase the resistance to CSF flow. For the construction of subject-specific models for the entire CNS, the spinal nerves connected to the spinal cord should be included to accurately capture CSF flow in the spine.
Chapter II. Effect of Trabeculae on Cerebral Spinal Fluid Flow and Drug Mixing

Abstract

**Background.** Spinal canal is filled with anatomical fine structures such as nerves and trabeculae which act as obstacles for cerebrospinal fluid flow (CSF) and drug distribution in the spinal canal. However, arachnoid trabeculae is below the imaging resolution of commonly used medical imaging modalities. The construction of computational models with thin trabeculae filaments allows for the investigation of arachnoid trabeculae on CSF flow and drug dispersion.

**Methods.** In this study, two computational models—one with no trabeculae and one with trabeculae—representing the spinal cavity were constructed. The effect of trabeculae on CSF flow and drug distribution was studied using computational fluid dynamics.

**Results.** In a constant flow, trabeculae increased CSF flow resistance 33%. However, flow resistance was not affect by trabeculae in a pulsatile flow. Trabeculae drastically increased drug dispersion speed.

**Conclusion.** CSF flow in the spinal canal has a pulsatile motion due to the expansions of cerebral vasculature at every heart beat. This study found that trabeculae do not significantly alter flow resistance in a pulsatile flow setting. However, the micro-eddies generated by trabeculae filaments accelerate drug dispersion.
Introduction

The subarachnoid space (SAS) is the space enclosed by the arachnoid and pia mater. The SAS is filled with cerebrospinal fluid (CSF). Besides from blood vessels, septa etc, there is a structure that gives a character of spider-web-like to SAS called trabeculae. The thickness of each trabeculae ranges from 5µm to 7µm. (11) Density of trabeculae varies, depending on which area of spinal cord and brain they are found. In a vault of human skull, arachnoid trabeculae join the adventitia to the arachnoid or pia mater or both, but the trabeculae proceeding directly from arachnoida to pia mater were not observed. (12) In optic nerve, in the bulbar segment (ampulla), a dense and highly ramified meshwork of delicate trabeculae is arranged in a reticular fashion. In the mid-orbital segment of the orbital portion, the subarachnoid space is subdivided, and can appear even loosely chambered by broad trabeculae and velum-like septa at some locations. In the intracanalicular segment additionally, few stout pillars and single round trabeculae are observed. (11) The arachnoid trabeculae are made up of an axis of connective tissue fibers all similarly oriented, and of one or more very thin, peripheral cytoplasmic layers showing a few interruptions, though they usually cover the fibers. (12)

Previous research involving trabeculae used near-infrared light propagation to study a pattern of light scattering in an adult head model. The researchers discovered that small amount of light scattering is caused by the arachnoid trabeculae in the CSF layer. The light that propagates in the CSF layer was, in practice, likely to be scattered and absorbed by these arachnoid trabeculae. In head models with discrete scatterers, the spatial heterogeneity in the CSF layer was considered to be caused by the arachnoid trabeculae and anisotropic scattering in the arachnoid trabeculae. Although in practice the optical properties and density of the arachnoid trabeculae in the CSF layer are not known, it is feasible that the volume density might range from 10% to 20% in an adult head. (13) The trabeculae are suspected to have an effect on the delivering speed by increasing resistance to CSF flow since they form a network across the path of the flow.

Methods

Computational Models and mesh generation
To study for the effect of trabeculae on resistance to fluid flow, a computational model with trabeculae at the density of 1 trabeculae per 0.25 cm³ was created. Another model without trabeculae was used as a control. These two models are shown in Figure 8. The density of trabeculae was estimated, since the actual density of trabeculae is not known. The dimensions were 5 cm × 1 cm × 0.3 cm. The model represents a CSF-filled domain within the spinal subarachnoid space filled with trabeculae.

![Figure 8. Geometry of the three-dimensional trabeculae model (left) and control model (right) constructed for the study of the effect of trabeculae on CSF flow resistance. Model dimensions are length = 5 cm, width = 1 cm, and height = 0.3 cm.](image)

<table>
<thead>
<tr>
<th>Geometry</th>
<th>Number of mesh</th>
<th>Length</th>
<th>Height</th>
<th>Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>270000</td>
<td>5 cm</td>
<td>0.3 cm</td>
<td>1 cm</td>
</tr>
<tr>
<td>2</td>
<td>894180</td>
<td>5 cm</td>
<td>0.3 cm</td>
<td>1 cm (spinal canal) 6 µm (trabeculae)</td>
</tr>
</tbody>
</table>

For studying the effect of trabeculae on drug biodistribution, an injection site was added in the center of the model with an opening diameter of 0.003 cm as shown in Figure 9. These models have the properties shown in

<table>
<thead>
<tr>
<th>Geometry</th>
<th>Number of volumes</th>
<th>Length</th>
<th>Height</th>
<th>Trabeculae</th>
<th>Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>508820</td>
<td>5 cm</td>
<td>0.3 cm</td>
<td>None</td>
<td>1 cm</td>
</tr>
<tr>
<td>2</td>
<td>579456</td>
<td>5 cm</td>
<td>0.3 cm</td>
<td>Diameter of 6 microns</td>
<td>1 cm (spinal canal)</td>
</tr>
<tr>
<td>Spinal canal</td>
<td>At 4 trabeculae per cm³</td>
<td>6 µm (trabeculae)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------</td>
<td>------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3 cm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(trabeculae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 9.** Computational meshes for the trabeculae model (left) and control (right).

**Governing equations and numerical methods**

The bulk fluid is assumed to be a Newtonian fluid which is incompressible and having constant viscosity. The simulations were done using commercial software, FLUENT. The calculation was based on the Navier Stokes equation, continuity and species transport.

**Boundary conditions**

Constant flow. Pressure drop across models with a constant flow was solved with a flow velocity of 0.01 m/s. Simulations were performed in steady-state.

Pulsatile flow. Pressure drop across models with pulsatile CSF flow was simulated by using a sinusoidal function with a frequency of 1Hz as shown in Equation 6. The pulsatile flow simulations are dynamic simulations instead of steady-state. Detailed instructions are included in Appendix 3.

\[
V=0.01 \sin(6.2318t) \tag{6}
\]

Species transport under pulsatile flow. Species are injected at the injection site with velocity of 4.71 cm/s, and the mass fraction of the species was 0.005. Detailed instructions for simulations are shown in Appendix 3.
Table 6. Boundary name, zone name, and boundary conditions

<table>
<thead>
<tr>
<th>Zone</th>
<th>Boundary</th>
<th>Boundary Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabeculae surfaces</td>
<td>Wall</td>
<td>Zero diffusivity flux</td>
</tr>
<tr>
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<td>Gauge pressure = 0 Pascal</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
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<td>Pressure outlet</td>
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<tr>
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<td>Zero diffusivity flux</td>
</tr>
<tr>
<td>Lower wall</td>
<td>Wall</td>
<td>Zero diffusivity flux</td>
</tr>
</tbody>
</table>

Results

**Pressure drop in constant flow**

Changes in pressure drop in models with and without trabeculae for a constant flow were quantified. For the control model, the pressure drop across the model is 0.95 Pascal. Simulations of the trabeculae model showed a pressure drop of 1.27 Pascal. Trabeculae increased the pressure drop by 33.7% in constant flow. The increase in pressure from the inlet to the outlet in both models is shown in Figure 10.
Pressure drop in pulsatile flow

During pulsatile flow, pressure drop changes dynamically, as shown in Figure 11. Trabeculae did not significantly increase the pressure drop of the oscillatory fluid flow. The pressure drop in the trabeculae and control models was about the same.

Drug Dispersion

The effect of trabeculae on drug dispersion was studied with a pulsatile fluid flow to mimic the effect of pulsating CSF in the spinal canal. Two injection methods were used. First, drugs are released at the flow inlet boundary with a constant concentration. Second, drugs are injected at the center injection site. Results show that trabeculae accelerates drug distribution during pulsatile flow, as shown in Figure 12.
Discussions

The spinal canal as well as the cranial subarachnoid space are filled with thin arachnoid trabeculae of about 6 microns thick connecting the arachnoid and the pia mater. Previously, the effects of the arachnoid trabeculae on drugs injected intrathecally are unknown. In order to predict accurate drug distribution after an intrathecal injection, we aim to reproduce the phenomenon of drug dispersion inside the spinal canal after a bolus injection of an anesthetic in our computational model. We hypothesize that thin trabeculae may cause enhanced drug mixing in the spine.

Drug distribution inside the spinal canal was simulated with a model filled with trabeculae and a control model without trabeculae. Our simulations show that the existence of the arachnoid trabeculae enhances drug mixing in the spinal canal. In the control model, the cerebrospinal fluid flow is laminar due to the absence of fine anatomical structures. In the model with trabeculae, the laminar flow is disrupted by these
thin filaments. Small eddies around these fine filaments are expected to be the reason of increased dispersion of the injected drug. However, since the CSF velocity is low, and the computed Reynolds number is still well below the turbulence region, the CSF within this anatomically complex space cannot be characterized as turbulence. The oscillatory flow phenomenon and the calculation of Reynolds number for pulsatile flows have been previously described by Mittal et al(14) and Watson.(15) Cerebrospinal fluid pulsations within a complex anatomical space may be characterized as a mixed flow phenomenon, which is the transition between laminar flow and turbulent flow.

It may be surprising that pressure drop during pulsatile flow is not greatly affected by the presence of trabeculae. Since the pressure drop of a fluid flowing through an obstructed domain is related to energy loss by wall shear stress, pressure drop is related to the total surface area of the obstructions within the domain. It may therefore be possible that the total surface area of the trabeculae introduced in this model was very small, and that they only cause a small increase in pressure drop. Increasing the density of the trabeculae, therefore increasing the surface area, is expected to generate a more significant difference in pressure drop during pulsatile flow.

Conclusion.

Arachnoid trabeculae increased the pressure drop by 33.7% in constant flow. However, they have a minimal impact on pressure drop and flow resistance during pulsatile flow. Micro-eddies are seen near these thin trabeculae filaments, and they disrupt laminar flow patterns. Arachnoid trabeculae increased drug dispersion inside the spinal canal, possibly through the disruption of laminar flow.
Chapter III. Generation of patient-specific computational models.

Abstract

Background. Mathematical principles can be used to compute fluid flow and drug distribution in computational domains. In order to investigate fluid mechanics and drug biodistribution in the central nervous system (CNS) for a specific subject, a patient-specific model needs to be reconstructed from medical images. This section will demonstrate the image reconstruction process of the human CNS based on magnetic resonance imaging.

Methods. High resolution medical images are loaded into a reconstruction software, MIMICS. Automatic thresholding and manual segmentation are used to select the patient's cerebrospinal fluid (CSF) filled subarachnoid space in the spinal canal and in the cranium. Special attention is paid to the reconstruction of anatomical details including the CSF-filled ventricles, brain sulci, the CSF spaces around the falx cerebri and tentorium, as well as the spinal nerve roots. The 3D object was converted into a 3D computational mesh. Meshing problems such as overlapping elements, holes, intersecting elements were fixed manually to produce an error-free computational mesh.

Results. This report contains results for anatomically accurate image reconstruction of the CSF space inside human CNS using medical imaging data. This mesh of the human CNS captures fine anatomical details such as the sulci, cerebral tentorium, pairs of nerve roots in the spinal canal, and current literature shows that this is the most detailed anatomical reconstruction of the human CNS to date.

Conclusion. The reconstruction of the human CNS was converted into a computational model which can be used for computational simulations of biofluid mechanics, drug infusion tests, and drug pharmacokinetics in the CNS.
Introduction

High resolution medical imaging provides opportunities for the generation of patient-specific models. In this chapter, a technique termed image reconstruction is introduced to create models that have subject-specific geometry. For predicting drug biodistribution in intrathecal drug delivery, the flow of the cerebrospinal fluid (CSF) within the complex spinal and cranial subarachnoid spaces needs to be modeled accurately. For building a complete model of CSF flow in the entire human CNS, the subarachnoid spaces within the human spine and brain were reconstructed with the image reconstruction techniques.

Methods

The three dimensional reconstruction is completed by using the image reconstruction software MIMICS, with MRI data obtained in accordance with guidelines. In MIMICS, automatic along with manual mask generation options were used to highlight the CSF filled space within each MRI image and build the three dimensional object that represents the CSF filled area inside the brain and spinal canal. Special attention was paid to reconstruct the CSF filled geometry in an anatomically consistent manner such as the ventricles, sulci (including the Sylvian fissures) as well as anatomical structures that obstruct and divert the CSF flow such as the falx cerebri and the tentorium (see Figure 13).

Figure 13. This figure shows the falx cerebri and the tentorium. Both consist of dura mater, the falx cerebri
A surface mesh representing the CSF filled spaces in the brain was then created in MIMICS using the “Remeshing” panel. The holes in the crude surface mesh were patched by creating surface triangles manually or by the automatic “fill” tool to create a continuous and closed volume representing the CSF space. The “wrapping” operation was performed to rid the crude surface mesh of small holes. The “smoothing” operation was performed to eliminate undesirable sharp features and contours in the surface mesh. The “quality preserving reduce triangles” operation was performed to minimize the number of surface triangles while preserving the quality and geometry of the mesh. The volume mesh necessary for drug transport simulations will be constructed based on the surface triangles of this surface mesh. Reducing the number of surface triangles on the surface mesh is a necessary step to reduce the number of elements in the volume mesh in the future to promote computational efficiency.

The reconstruction of the spinal canal was performed under my supervision by IMSA fellow Candice Yi. With MRI image data, automatic and manual mask generation options were used to produce the mask representing the CSF filled spaces inside the spinal canal. The surface mesh was then created by via the “Remeshing” panel. The operations done on the surface mesh to improve its quality were similar to the operations described in the previous paragraph.

The computational meshes representing the CSF filled spaces in the human brain and the spinal canal were then fused to produce a continuous and closed volume by using the function “Boolean operations” then “Unite” in MIMICS.

Results

The image reconstruction results (shown in Figure 14(a)-(d)) capture the complex CSF filled spaces in the CNS of human. Previous models only included the CSF around the cerebral cortex, cerebellum, and the ventricles, but left out many other anatomical details. This model features the CSF filled sulci (Figure 14(b)) as well as the CSF spaces around the falx cerebri and the tentorium (Figure 14(c)).

The reconstruction of the CSF space inside the spinal canal from MRI imaging data also features high level of detail in anatomical accuracy. In humans, there are 31 pairs of nerves protruding outward from the spinal cord. In human CSF space inside the spinal canal, these nerve roots leaving the cord are obstacles to the CSF flow inside the
intrathecal space (Figure 14(a)). The inclusion of these nerves may contribute to more accurate prediction of the CSF flow pattern and drug distribution prediction.

Figure 14. Reconstruction of the subject’s subarachnoid space in the brain and spine. (Top-left) The entire CSF-filled subarachnoid space in the human CNS, inset shows the delineation of the spinal nerves. (Top-right) CSF-filled sulci in the brain. (Bottom-left) Posterior view of the cranial CSF space shows the tentorium and falx cerebri. (Bottom-right) A coronal section of the CSF-filled subarachnoid space in the brain.
Discussion

This computational mesh of CSF filled spaces preserves many anatomical details inside the human brain, in order to accurately define the mechanics of CSF flow. The falx cerebri is film-like dura mater that descends from the superior sagittal sinus to the inferior that separates the left and right hemisphere. Cerebrospinal fluid descends on both side of the falx cerebri and the two sheets of CSF fluid unite right above the corpus callosum. The tentorium is also formed with dura mater separating the cerebro cortex with the cerebellum. The tentorium forms a barrier obstructing CSF flow at the medial-dorsal part of the brain. The existence of the falx cerebri and tentorium increase the resistance of CSF flow in the brain, because CSF has to diverge around these structures without a direct flow path. The incorporation of these anatomical details is expected to increase the accuracy of CSF flow simulations as well as drug transport simulations, as these anatomical structures are expected to heavily influence the CSF flow field. The inclusion of spinal nerves is necessary for the accurate prediction of CSF flow in the spinal canal.

The inclusion of the fluid-filled sulci may contribute to the accurate computation of drug distribution inside the CSF space. In the future, this model will lead to a better prediction of drug distribution into the grey and white matter, because the inclusion of the sulci structures promotes the accurate definition of the surface area of the cortex, and allows for diffusion of the drug into the brain tissue.

Conclusion

An anatomically consistent computational model representing the CSF filled spaces of the human brain and spinal canal was constructed based on MR imaging data.
Chapter IV. The frequency and magnitude of cerebrospinal fluid pulsations influence intrathecal drug distribution - key factors for interpatient variability

Abstract

Background: Intrathecal (IT) drug delivery is an efficient method to administer therapeutic molecules to the central nervous system (CNS). However, even with identical drug dosage and administration mode, the extent of drug distribution in vivo is highly variable and difficult to control. Different cerebrospinal fluid (CSF) pulsatility from patient to patient may lead to different drug distribution. Medical image-based computational fluid dynamics (miCFD) is used to construct a patient-specific model to quantify drug transport as a function of a spectrum of physiological CSF pulsations.

Methods: Magnetic resonance imaging (MRI) and CINE MRI were performed to capture the patient’s CNS anatomy and CSF pulsatile flow velocities. An miCFD model was reconstructed from these MR images and the patient’s CSF flow velocities were computed. The effect of CSF pulsatility - frequency and stroke volume - was investigated for a bolus injection of a model drug at the L2 vertebral level. Drug distribution profiles along the entire spine were computed for different heart rates: 43bpm, 60bpm and 120bpm, and varied CSF stroke volumes: 1ml, 2ml, 3ml. To assess toxicity risk for patients with different physiological parameters, therapeutic and toxic concentration thresholds for a common anesthetic were derived from experimental studies. Toxicity risk analysis was performed for an injection of a spinal anesthetic for patients with different heart rates and CSF stroke volumes.

Results: Both heart rate and CSF stroke volume of the patient strongly influence drug distribution administered intrathecally. Doubling the heart rate (from 60bpm to 120bpm) caused a 26.4% decrease in peak concentration in CSF after injection. Doubling the CSF stroke volume diminished the peak concentration after injection by 38.1%. Computations show that potentially toxic peak concentrations due to injection can be avoided by changing the infusion rate. Using slower infusion rates could avoid high peak concentrations in CSF while maintaining drug concentrations above the therapeutic threshold.

Conclusions: Our computations identify key parameters for patient to patient variability in drug distribution in the spine observed clinically. The speed of drug transport is strongly affected by the frequency and magnitude of CSF pulsations. Toxicity risks associated with an injection can be reduced for a particular patient by adjusting the infusion parameters with our rigorous miCFD model.
Introduction

Intrathecal (IT) drug delivery is an efficient way to administer therapeutic molecules to the central nervous system (CNS). However, IT infusions even with identical dosage during controlled settings lead to high inter-patient variability in drug distribution. Factors that influence IT drug distribution are numerous, including anatomical abnormality, patient posture, infusion rate, catheter diameter, lumbrosacral CSF volume, CSF densities, and CSF pressure. Actual rates of drug binding and uptake in the spinal cord tissue of different subjects as a function of subject-specific receptor density or abnormal receptors may also affect drug transport and in effect biodistribution.

In this chapter, a novel method, medical image-based computational fluid dynamics (miCFD), was used for investigating IT drug delivery. miCFD combines quantitative medical imaging and computational fluid dynamics (CFD) to generate patient-specific computational models. The method has four stages: First, observe and measure in vivo CSF flow phenomenon. Second, build a model of the patient’s spinal cord and subarachnoid space through image reconstruction, assign realistic tissue properties and recreate CSF flow velocities. Third, validate the model with in vivo flow measurements. Fourth, compute concentration profiles for a desired infusion and optimize IT infusion parameters for this particular patient. The miCFD method generates a computational model of the subarachnoid space and spinal cord with accurate anatomical shape in two or three dimensions, as well as the dynamics of the patient’s CSF pulsations. This patient-specific model can generate quantitative predictions of drug distribution for an infusion with desired parameters such as injection concentration, flow rate, duration, patient position, solution baricity and infusion site. The miCFD model outputs drug biodistribution in space and time, so the local drug concentrations at a region of interest in the subarachnoid space or spinal cord tissue can be monitored continuously to avoid toxicity risk.

We will predict spatiotemporal drug concentration profiles for an IT infusion and quantify the effect of two critical physiological parameters: heart rate and CSF stroke volume. Heart rate and CSF pulsatile magnitudes in the spine vary greatly from patient to patient. CSF pulsates inside the spinal canal at the frequency of the heart beat. With a surrogate spine model, Hettiarachchi et al. found pulsating CSF in the spine as a main driver for drug distribution. These CSF pulsations cause additional mixing and dispersion of intrathecally infused molecules compared to pure diffusion alone in a stagnant fluid. We hypothesize that variations in CSF pulsatility (frequency and stroke volume) may lead to inter- and intra-patient variability in drug distribution. We therefore test whether
these key factors contribute to the high variability in drug distribution observed clinically. The miCFD model serves as a platform for simulated infusion tests to enhance the fundamental understanding of the hydrodynamics and drug transport in the subarachnoid space and spinal cord tissue.

This study has three principle components: 1) CINE MRI measurements of CSF flow velocities and the computation of CSF flow velocities for a particular patient; 2) Simulation of IT infusion of a model drug and quantification of the effects of heart rate and CSF stroke volume; 3) Toxicity risk analysis for the infusion of a common spinal anesthetic with experimentally derived therapeutic and toxicity thresholds.

Methods

Data acquisition of CINE-MRI imaging was performed by Dr. David Zhu in the University of Michigan. CINE (or time resolved) MRI was used to obtain images with cardiac gating to trigger data acquisition.(27) The full image data set is acquired through multiple cardiac cycles and is linearly interpolated to a data set as acquired from the same points of a single cardiac cycle. A two-dimensional CINE phase contrast technique was applied to collect CSF flow data from the spinal canal of a 29-year old healthy male on a 3T Signa® HDx MR scanner (GE Healthcare, Waukesha, WI) equipped with a high-density CTL spine coil. The volunteer signed the consent form of research agreement that had been approved by the Institutional Review Board at Michigan State University.

One hundred axial $T_2$-weighted fast spin echo (FSE) images with a time of echo (TE) = 102 ms, a time of repetition (TR) = 5300 ms, echo train length = 8, receiver bandwidth (rBW) = 31.25 kHz, image field of view (FOV) = 12 cm, slice thickness = 5 mm, matrix size = 256 × 256 and number of excitation = 2 were collected at the upper and then another 100 images at the lower part of the spinal cord. The two hundred images were combined together to provide a high-resolution view of the full spinal cord. Then CINE phase contrast images were acquired in axial direction at locations of interest. Velocities in all three directions were measured to investigate the flow dynamics based on the simple four-point method.(28) Flow compensation and peripheral gating were applied. A low maximum velocity (VENC) of 5 cm/sec had been chosen as the limit so that a reasonable velocity resolution could be achieved for CSF flow measurement. Other acquisition parameters were: TE = 9.5 ms, TR = 29 ms, flip angle = 20°, rBW = 15.6 kHz, FOV = 12 cm, slice thickness = 5 mm, matrix size = 256 × 256, and number of excitation = 2. Sixteen images were reconstructed per cardiac cycle.

The CSF pathway was manually segmented based on the $T_2$-weighted FSE image acquired in which CSF was enhanced. The velocity of every pixel at regions of CSF was calculated. To reduce the possibly spatially dependent
offset velocity due to eddy currents or motion of the body, the velocity at each pixel location was corrected through basic subtraction by the time-average “velocity” of a nearby solid spinal cord or muscular tissue “background” within a 19mm × 19mm region with this pixel at the center of this “background”. (29-31) Solid tissue does not accumulate net displacement over a complete cardiac cycle. (29-31)

**Computation of CSF flow in the CNS**

An image reconstruction software, MIMICS*, was used to construct the patient-specific miCFD model from MR images of the CNS as described in chapter III. Computation of CSF flow was performed by applying the continuity and Navier Stokes equations as described in chapter I. The CSF pulsations with arterial waveform (32) was emulated by periodic functions (equation 3):

\[
\bar{u}(t) = a_0 + \sum_{n=1}^{N} \left( a_n \cos n\omega t + b_n \sin n\omega t \right)
\]

(1)

where \( \bar{u} \) is the periodic velocity field.

This miCFD model accounts for physiological CSF production, reabsorption and pulsations (Figure 15). A summary of model properties is shown in Table 7. The production of CSF by the choroid plexus at 0.4ml/minute was implemented by an inflow on the walls of the lateral and third ventricles. (33) The reabsorption of CSF occurred in the superior sagittal sinus as an outflow. The pulsatile CSF flow was modeled by imposing pulsatile CSF velocities at the surface of the cortex and ventricular walls with a cardiac waveform. (32) The CSF pulsatile velocities were validated with in vivo measurements. More details on the computation of the CSF flow field can be found in literature. 17, (34)

Simulation of drug distribution during an intrathecal infusion

For the respective quantification of the effects of heart rate and stroke volume, a 0.2ml bolus injection of a model drug over 30 seconds was simulated at L2. The injected solution was isobaric. Drug transport by diffusion and convection in CSF is modeled by the species transport equation as shown in chapter II.

Drug diffusion inside the spinal cord was computed by Darcy's law (equation 5):

$$\varepsilon \frac{\partial C}{\partial t} + \vec{u} \cdot \nabla C = \nabla \cdot (D \nabla C)$$  \hspace{1cm} (5)

where the porosity of tissue is set to $\varepsilon = 0.3$. The permeability of the tissue is $1.0 \times 10^{-16}$. Drug concentrations at 1mm and 2mm beneath the dorsal surface of the cord were taken to study drug perfusion into the spinal tissue at different vertebral levels.

**Independent effects of heart rate and CSF stroke volume**

The patient's heart rate is the frequency of CSF pulsations in the spine. CSF pulsatile stroke volume is the amount of CSF displaced during each pulsation at the cervical spine. Drug distribution was computed for three different heart rates of 43, 60 and 120 bpm. Independently, CSF stroke volume was set to 1.0, 2.0, and 3.0ml to observe its effect on drug distribution. Other infusion parameters were held constant.

Infusion of a local anesthetic

Figure 15 (A). Boundary conditions of the computational model. 1(B) Comparison between computed CSF velocities at C4, CINE-MRI measurements, and published values (at C3-4 level). 1(C) Injection and sampling sites in CSF and tissue near the L2 vertebral level.
Infusion of 2ml of 10mg bupivacaine hydrochloride (0.5% solution) at the L2 vertebra over 30 seconds was simulated using a common infusion protocol\(^{(38)}\) to study the combined effect of heart rate and CSF stroke volume on drug biodistribution and potential toxicity risk.\(^{(38)}\) The solution is isobaric. The onset of anesthesia and the toxicity of local drug concentrations in the spine were analyzed with threshold values for concentrations of local anesthetics identified by Post and Freedman.\(^{(39)}\)

Table 7. Boundary conditions of the computational model

<table>
<thead>
<tr>
<th>Model properties</th>
<th>Anatomical location</th>
<th>Values</th>
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<tbody>
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<td>CSF production</td>
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<td>CSF production rate(^{(40-41)}) = 0.4(ml/min)</td>
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<tr>
<td>CSF reabsorption</td>
<td>Arachnoid granulations</td>
<td>CSF outflow(^{(40)})</td>
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<td>CSF pulsations</td>
<td>Prefrontal cortex surface</td>
<td>Periodic velocity waveform((\text{m/s}))</td>
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<tr>
<td></td>
<td>Ventricular walls</td>
<td>Periodic velocity waveform((\text{m/s}))</td>
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<td></td>
<td>Occipital cortex surface</td>
<td>Periodic velocity waveform((\text{m/s})) with a phase lag</td>
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<td>Tissue properties</td>
<td>Spinal cord</td>
<td>Porosity(^{(42)}) = 0.3</td>
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<tr>
<td></td>
<td></td>
<td>Mean viscous resistance(^{(42)}) = 1x10(^{-16})(1/m(^2))</td>
</tr>
</tbody>
</table>

| Infusion protocol 1 | Drug: model drug                      | Molecular weight = 213.6(kg/kmol)                                   |
|                    | Injection site: L2                   | Concentration = 500(µg/cc)                                          |
|                    |                                       | Mean effective diffusivity\(^{(43)}\) = 2.1x10\(^{-10}\)(m\(^2\)/s)  |
|                    |                                       | Infusate baricity = 1                                                |
|                    |                                       | Bolus infusion dosage = 100(µg)                                      |
|                    |                                       | Bolus infusion duration = 30(s)                                      |

| Infusion protocol 2 | Drug: spinal anesthetic              | Molecular weight = 324.89(kg/kmol)                                  |
|                    | Injection site: L2                   | Concentration = 5(mg/cc)                                             |
|                    |                                       | Mean effective diffusivity\(^{(43)}\) = 2.1x10\(^{-10}\)(m\(^2\)/s) |
|                    |                                       | Infusate baricity = 1                                                |
|                    |                                       | Bolus infusion dosage = 10(mg)                                       |

Results

CSF velocities at C4, T6 and L4 in the spine were measured. Figure 16 shows CSF velocity magnitudes in axial images of the spine during systolic and diastolic CSF flow (velocity magnitudes greater than 3.0cm/sec are indicated in red). Figure 16 (c) compares measured CSF velocities at C4, T6 and L4. Positive direction indicates systolic CSF flow. The peak systolic CSF velocity is 2.5cm/s, while the peak diastolic velocity is 2.2 cm/s. Systolic CSF flow has a larger amplitude than diastolic CSF flow.
Figure 16. CINE-MRI measurement of cerebrospinal fluid (CSF) flow velocities at C4, T6 and L4.  
2(A). Peak systolic (caudal) CSF velocities at C4, T6, and L4 in a volunteer.  The volunteer is lying on his back, facing up.  Red shows CSF velocity magnitudes greater than 3.0 cm/sec, while green shows approximately zero velocity.  2(B) Peak diastolic (rostral) velocity at C4, T6 and L4.  2(C) Measured CSF velocities over one cardiac cycle.  Positive velocities are in the direction of systolic flow.  The peak systolic CSF velocity is 2.5 cm/s, while the peak diastolic CSF velocity is 2.2 cm/s.  The sagittal image of the volunteer’s spine shows three locations of velocity measurement.  The white lines mark the axial planes where CSF velocities were measured.  The label indicates the subarachnoid space filled with CSF and its nearest vertebral level.

<table>
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<td><img src="image7" alt="Image of L4" /></td>
<td><img src="image3" alt="Graph of CSF velocities" /></td>
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</table>

The miCFD image reconstruction captures the anatomical dimensions of the subarachnoid spaces and the spinal cord of the patient in the MRI.  The equivalent total CSF volume of our patient's two-dimensional computational model - assuming rotational symmetry - amounts to 232.38 ml.  The total vertebral CSF volume of the model is 122.27 ml.  The CSF volume below T11 vertebra to the end of dural sac in the model is 53.03 ml.  The reconstructed
compartmental CSF volumes agree with reported experimental measurements on healthy subjects. For example, Courchesne et al. (44) reported intracranial CSF volume to vary between 50ml to 480ml between 116 healthy volunteers. Total vertebral CSF volume (measured in two normal subjects) reported by Hogan et al. are 94.8ml and 120ml, respectively. (45) The mean lumbrosacral CSF volume (bellow T11-T12 vertebrae, including the nerves roots) was found to be 49.9ml. (45) The structural dimensions of the reconstructed subarachnoid space are unique to this specific patient. These specific biometrics determine the maximum volume of dilution for drugs infused into the spine.

CSF flow patterns including pulsations, production and reabsorption were reproduced in the miCFD model. CSF stroke volume at the cervical spine was 1.5ml. The comparison between measured and computed flow velocities at upper cervical region are shown in Figure 16. At C4, the computed peak systolic and diastolic CSF flow velocities are 3.4cm/s and 2.5cm/s, respectively. Henry-Feugeas et al. (35) reported that the ventral CSF systolic velocity was 3.5cm/s, diastolic velocity was 2.3cm/s, and the diastolic velocity magnitude was 71% of the systolic velocity magnitude at C3-4. In the miCFD model, systolic and diastolic CSF flows account for 45% and 55% of the cardiac cycle, respectively. Measured CSF systolic and diastolic flows at C4 last 44% and 56% of the cardiac cycle, respectively. (35) Computed systolic peak velocity has larger amplitude than the diastolic peak velocity, and the systolic flow duration is shorter than the diastolic flow duration consistent with physiological data. The phase lag between local CSF pulsations in the pontine cistern and aqueduct caused by the precise timing of the vasculature expansions as measured by Henry-Feugeas et al. (35) was also captured consistently in the model.

After IT injection of a model drug for a patient with heart rate of 60bpm and CSF stroke volume of 1.5ml, concentration in CSF and spinal cord tissue was taken for 120 minutes (Figure 17). Drug concentration is normalized to the injection concentration, showing the percent dilution. Temporal evolutions of drug concentrations are shown at six locations in the spine (C4, T4 and T10 in CSF and in the spinal cord, respectively). Figure 17 shows that after injection, drug concentration in CSF at T10 rose and reached a maximum after 8 minutes. Drug concentration declined rapidly due to dilution afterwards. The computed distribution half-life of the drug is about 45 minutes. (Sallerin-Caute et al. (46) reported distribution half-life of baclofen administered as a bolus at T9-T10 to be between 0.947-1.04 hour.) Drug concentration 2mm within the spinal cord tissue at T10 reached a maximum after 63 minutes, 55 minutes later than the maximum in adjacent CSF, and declined slowly due to diffusion. Drug concentration in CSF at C4 reached a maximum 30 minutes after injection, and the concentration
was diluted to half-maximum 90 minutes after injection. The concentration peak in the spinal cord tissue (2mm deep at C4) arrived 65 minutes after the CSF (C4) concentration peak due to slow diffusion in the tissue. 

As the drug concentrations in CSF rose after injection, CSF pulsations induce rapid mixing leading to rapid decline of concentration peaks. Due to mass transfer and diffusion, drug levels reached a peak in the spinal cord tissue approximately an hour later than the peak in the surrounding CSF at that vertebral level. In Figure 17, drug concentrations within the spinal cord tissue rose above drug concentrations in the adjacent CSF after about 40 minutes. After infusion, the rapid mixing in the pulsating CSF caused dilution of the drug in the subarachnoid space. Note that drug concentrations in the extracellular space may even temporarily remain above those found in the adjacent CSF. These levels are due to the capacity of the tissue to hold previously accumulated drug. The slow drug diffusion and dilution in the spinal cord tissue are responsible for the observed drug accumulation.

**Heart rate.** Drug distribution was investigated with heart rates of 43, 60 and 120bpm. After injection, peak concentrations were found near L2 in the CSF. Figure 18 shows that peak concentrations at L2 after injection for 43, 60, 120bpm reached 1.1%, 0.91%, and 0.67%, respectively. In comparison, Sallerin-Caute et al.(46) reported peak baclofen concentration in CSF to be 1% of the injectate near the injection site in a clinical study. The virtual patient with the slowest heart rate (43bpm) resulted in the highest drug concentration peak after injection at L2. When heart rate was elevated from 43 to 60bpm, the peak concentration near L2 fell by 17%. When the heart rate was elevated from 60 to 120bpm, the peak concentration near L2 fell by 26.4%.

**CSF stroke volume.** Drug distribution was computed for CSF stroke volumes of 1, 2, and 3ml. After injection, peak concentrations in CSF near L2 were 1.26%, 0.78%, and 0.57%, respectively (Figure 19). When CSF stroke volume was elevated from 1.0 to 2.0ml, thus increasing the CSF pulse magnitude, peak concentration after injection fell by 38.1%. When CSF stroke volume was elevated from 2.0 to 3.0ml, peak concentration fell by 26.9%.
**Infusion of a local anesthetic and toxicity risk analysis**

The simulated intrathecal infusion of 10mg bupivacaine - a spinal anesthetic - in 2ml of solution (0.5%) over 30 seconds is similar to the commonly used clinical protocol.(38) Toxicity risk analysis was performed comparing spatiotemporal concentration curves with therapeutic and toxicity thresholds found in the experimental studies. The *effective anesthetic concentration* in spinal cord tissue was inferred from experiments of radio-labeled bupivacaine infusion in mice by Post and Freedman.(39) After infusion, complete blockade in the hind-legs of mice were observed and the radioactivity in the axial spinal cord tissue slices (5mm thick) was measured to quantify bupivacaine concentrations in tissue at different vertebral levels along the spinal cord. Associating the experimentally measured bupivacaine concentrations in the lumbothoracic cord of the mice with the observed blockade, we derived the therapeutic threshold of bupivacaine for the prediction of onset time. Equally, the *toxicity threshold* of bupivacaine for our simulations was based on in-vitro measurements of the half-maximum inhibitory concentration that causes morphological changes - growth cone collapse and neurite degeneration - of dorsal root ganglion neurons due to direct exposure by Radwan et al.(47) To assess the difference in toxicity risks for patients with different physiological parameters, spatiotemporal bupivacaine concentrations in CSF and in spinal cord tissue were computed for two patients with different heart rate and CSF stroke volume (Fig 6). Patient 1 has heart
rate of 60bpm and CSF stroke volume of 1.5ml, and patient 2 has heart rate of 50bpm and CSF stroke volume of 0.6ml. Patient 2 has a lower CSF pulsatility than patient 1.

**Peak Concentration.** For patient 1, the infusion of 0.5% bupivacaine (10mg) over 30 seconds produced a local concentration peak in CSF near the injection site (L2) of 3.8mmole per ml of CSF, which is above the derived toxic threshold. Bupivacaine solution at this concentration was observed to produce degeneration in neurons at fifteen minutes exposure by Radwan et al.(47) Even though this peak concentration drops below toxic threshold after 100 seconds, brief toxic concentrations may potentially harm unprotected nerves, such as the nearby cauda equina.(48)

![Figure 20. Temporal evolutions of anesthetic concentration in cerebrospinal fluid (CSF) and tissue for patients with different heart rate and CSF stroke volume.](image)

![Figure 21. Infusion adjustments for risk reduction for patient 1. Patient 1 has heart rate of 60bpm and stroke volume of 1.5ml.](image)

**Onset.** Anesthetic concentrations in the spinal cord tissue could be correlated with the time of ensuing therapeutic onset. For patient 1, bupivacaine concentration in the spinal cord tissue at L2 (1mm depth from the dorsal tissue surface) rose reaching therapeutic window after about six minutes (325 seconds), as seen in Figure 20. Our computer simulations predicted onset time within the range of clinically reported values. Malinovsky et al.(38) infused 0.5% isobaric bupivacaine (10mg) in 15 patients at L2/L3 and reported that the onset time for pinprick test ranged from 3-29 minutes, for cold test ranged from 3 to 29 minutes, and for motor blockage ranged from 4 to 22 minutes, respectively.

**Comparison of patient 1 and 2.** Anesthetic concentrations in L2-CSF and L2-cord tissue (1mm from dorsal cord surface) were plotted for two patients. The CSF peak concentrations and time to peak concentrations are
3.84mmole/cm³, 73.55 seconds for patient 1 and 4.02mmole/cm³, 209.45 seconds for patient 2. Patient 2 has a significantly longer peak arrival time due to the low-frequency, low-magnitude CSF pulsations in the spine. An important observation is that the low CSF pulsatility in patient 2 lead to a 7-minute duration of potentially toxic concentrations in CSF at L2. Patient 2 has a higher risk than patient 1 associated with this administration mode of 10mg spinal anesthetic due to prolonged exposure of high concentrations.

*Infusion adjustment for reduced toxicity*

The brief concentration peak at the toxic level for patient 1 can be avoided by adjusting the infusion rate, while administering the same amount of anesthetic. We computed concentration profiles for different infusion rates to reduce the toxicity risk for patient 1. Four different infusion rates are tested where 10mg of bupivacaine in 2ml solution was injected over 0.5, 1.5, 5 and 7 minutes, from fast to slow infusion rates. Anesthetic concentrations in CSF and spinal cord are reported in Figure 21 for different infusion rates to compare peak CSF concentration, onset times, tissue concentrations and the associated toxicity. The trend shows that the peak concentrations in L2-CSF are reduced when slower infusion rates are used to deliver the same drug dosage. When the dose was administered over 5 and 7 minutes, no toxic peaks in CSF are present (line 3 and 4, Figure 21) at all times. For these slower infusion rates, onset times for the therapeutic effect of the anesthetic are delayed for about 4-5 minutes compared to the original 0.5 minute infusion. However, tissue concentrations are almost the same after 15 minutes regardless of the difference in infusion rates, which suggests that prolonged therapeutic effect may be conserved despite using slower infusion rates. By choosing slower infusion rates, local concentrations peaks in CSF that are potentially toxic can be avoided to minimize the exposure of nerve roots.

**Discussion**

IT drug distribution is a function of the frequency and magnitude of CSF pulsations. While resting heart rate vary from 50bpm-90bpm in normal subjects, a much wider range, from as low as 15bpm during bradycardia to as high as 180bpm during exercise, is possible.(49) In the patient specific model, a 100% elevation in heart rate (from 60bpm to 120bpm) caused a 26.4% decrease in maximum concentration in CSF after injection. The high CSF pulsatile frequency lead to faster drug dispersion, causing lower concentration peaks due to better mixing.
Inter-patient variability in CSF pulsatile magnitude is another critical parameter for IT drug distribution. Between normal subjects, maximum CSF velocity measured at the C4 ranged from 1.9cm/s to 5.1 cm/s. Variations in CSF velocity magnitudes in the spine correspond to different CSF stroke volumes. In this study, we found that different CSF stroke volumes lead to wide variations in dispersion patterns. High CSF stroke volumes cause more extended drug distribution along the spine. In comparison, low CSF stroke volumes lead to slower drug dispersion, causing high concentration peaks after injection.

Intra-patient variability in CSF stroke volume may exist as well. CSF pulse magnitudes correlate with blood pressure. This correlation has been observed by Hamer et al. (50), where a rise in systemic arterial blood pressure correlates with an increase in CSF pulse magnitude. A direct relation between the arterial waveform amplitude, venous waveform amplitude and CSF waveform amplitude is observed by Bhadelia et al. (32) A significant positive correlation was found between arterial waveform amplitude and CSF waveform amplitude; those subjects with high-amplitude arterial waveform also have high-amplitude CSF waveform. The amplitudes of arterial-venous waveforms are indicators of the amplitude of CSF waveform of the subject. Variations in CSF waveform amplitudes of 21 subjects in this study can be partially accounted for by their arterial and venous waveforms. Variations in arterial and venous blood flow between subjects are responsible for 56% of the variations in CSF pulsatile magnitude between these subjects. Other factors such as brain tissue compliance and total CSF volume are responsible for the rest of the variations in CSF pulse magnitude. During spinal anesthesia, the systolic blood pressure could be reduced by about 60 mmHg, and the diastolic pressure could decrease by about 20 mmHg. Blood pressure fluctuations during anesthesia can reduce CSF stroke volumes in the spine. We found that these physiological conditions of a patient can influence infusion outcome by means of CSF pulsatility in the spine.

We have performed infusion experiments with a radio-tracer in a surrogate spine model and quantified the tracer distribution with single positron emission computed tomography (SPECT) to validate our computational model of the spine. Results shows a good match between the concentrations of the tracer measured by SPECT and the concentrations reported by the computational model. The CSF velocity and drug concentrations reported by our miCFD model are compared with clinical measurements. The CSF velocity magnitudes, ratio of systolic and diastolic peak velocities and durations of systolic and diastolic flows agree with clinical measurements by Henry-Feugeas et al. (35) and our CINE-MRI measurements. For validation of drug infusion, peak drug concentration in CSF after injection was compared with clinical studies. For heart rates of 43, 60, and 120 bpm, the miCFD model reports peak concentrations in CSF after injection of 1.1%, 0.91%, and 0.67%, respectively. Comparably, Sallerin-
Caute et al.\textsuperscript{(46)} reported peak baclofen concentration in CSF to be 1\% of the injectate near the site of injection in a clinical study. Our miCFD model generates theoretical estimates of drug biodistribution in the spine.

We have demonstrated that the difference in patient physiological parameters may cause certain patients to be more susceptible to toxicity risk associated with the infusion of an anesthetic. This may explain the high variability in infusion outcomes observed clinically for spinal drug administration. It may be conjectured that patients with very low CSF pulsatility could suffer unexpected side effects for a seemingly safe and uneventful procedure.\textsuperscript{(52-54)} To reduce toxicity risk associated with an injection, infusion parameters can be adjusted for a particular patient based on the patient's physiological conditions as well as the spinal anatomy.

The rational design of personalized infusion therapies using miCFD can reduce the risk associated with spinal drug administration. This study shows that the \textit{local} concentration of the anesthetic near the neuronal tissue is more significant for assessing neurotoxicity as opposed to merely considering infusate concentrations. Detailed computations permit rigorous toxicity risk analysis based on spatiotemporal evolution of \textit{local drug concentrations} at selected sites of interest. \textit{In vitro} experiments on isolated nerve exposure\textsuperscript{(55-57)} or \textit{in vivo} animal experimentation\textsuperscript{(39)} would provide the toxicity thresholds for these patient-specific simulations. 'Safe' infusions would thus ensure local drug concentrations in space and time below toxicity threshold at all times during the procedure, even if the patient's physiological parameters change over time. Even brief exposures of unprotected nerve roots to high concentrations could thus be avoided.

The proposed method offers a rigorous approach to generate the likely outcomes of an infusion.

However, limitations in imaging resolution and incomplete pharmacodynamic knowledge warrant caution in adopting the theoretical predictions in the clinical practice. Even though the CNS anatomy is captured in the model, fine structures such as nerve roots, septum and trabeculae in the subarachnoid space are not reconstructed in the present model. Future investigations will quantify the effect of these fine structures on drug dispersion. The emphasis of the current study is on drug transport as a function of CSF pulsatility, while biochemical reactions of the specific drug have not yet been included. Due to the coupling of reactions and transport, drug binding, endocytotic uptake and degradation will affect drug biodistribution. Drug-specific biochemical properties can be incorporated into the current model as described in Linninger et al.\textsuperscript{(42)} to investigate drug interaction with receptors and cells at the target site.
Conclusions

The advancement in quantitative medical imaging technologies enable the construction of anatomically and physiologically consistent models. The computation of drug distribution based on advanced medical imaging and patient-specific parameters gives new insight into the cause of high variability in drug distribution in the spine. Patient physiological parameters such as heart rate and CSF stroke volume were found to be key factors in drug biodistribution. Our miCFD model enables the performance of infusion tests on the computer with desired infusion parameters. Its potential applications include predicting drug distribution for different infusion rates, locations, and concentrations, as well as designing infusion protocols for patients with obstruction or anatomical abnormality in the spinal canal captured by MRI. Quantitative and systematic investigation using miCFD will enhance the fundamental understanding in drug transport, distribution and the optimization of therapeutic outcome. IT drug administration has immense potential for administering therapeutics into the CNS such as nanoparticles, neurotrophic factors, and gene-based therapy. With rational design methods, toxicity risks associated with spinal drug administration can be minimized and the design of personalized infusion therapies is possible.
Chapter V. The development of single-cell kinetic model for gene silencing therapies.

Abstract

Background. Gene silencing is a novel mechanism of the translational repression of a target gene based on RNA interference. The injection of short-interfering RNAs downregulates the expression level of a particular gene without modifying genetic information. The RNA interference (RNAi) pathway involves the complimentary-binding of siRNA to specific mRNA within the cell and the recruitment of an RNAi protein complex, leading to translational repression. For the rational design of human gene silencing therapies, we have constructed a mathematical model of RNA interference in cells. The model computes concentrations of proteins involved in the RNA interference network. In the next chapter, this model will be combined with the image reconstruction of the human spinal cord for the rational design of infusion therapies targeting chronic pain.

Methods. The RNA interference mechanism is modeled with partial differential equations in MATLAB. The fate of siRNAs in cells including endocytosis, unpackaging from endosomes, binding to RNA-induced silencing complex, and the targeting and degradation of messenger RNA were modeled. In addition, a co-infusion design to enhance gene silencing efficacy was simulated by the infusion of a clathrin antibody to inhibit clathrin-mediated endocytosis in untargeted cells.

Results. In MATLAB, integration of partial differential equations gives predicted temporal trajectories of the target receptor level in response to the administration of siRNA. Infusion dosage and duration could be adjusted to achieve optimal gene silencing.

Conclusions. The mathematical modeling of gene silencing efficiency in cells can predict the levels of receptor downregulation after the administration of siRNAs.
Introduction

Gene silencing therapies are the next generation of treatments for CNS disorders. siRNA molecules bind and destruct target mRNA in the cell, changing the expression levels of the target protein at the cellular level. This promising technique induced down-regulation of NMDA receptor subunit NR2B in the spinal cord of rats, temporarily inhibiting pain transmission. The NMDA receptor mediates sensitized pain in chronic pain subjects. A design method is proposed in this article to precisely control the expression of disease-related proteins in the human CNS. High resolution medical imaging can capture fine anatomical details. The integration of medical reconstruction with mathematical models may provide quantitative answers to issues that currently delay the clinical implementation of human gene silencing therapies. In this chapter, a mathematical model representing gene silencing kinetics in neuronal cell cultures is constructed to describe the dose-response relationship of siRNA concentration and NMDA receptors downregulation. In the next chapter, this cellular model will be fused with medical image reconstruction, biotransport, and region-specific siRNA bioreactivities to generation dose-response predictions for the entire spinal cord. Our goal is to translate bench-top results towards the clinical implementation of gene silencing therapies. Computational prediction of RNA interference can generate optimal infusion dosage of siRNA using a patient-specific geometry.

Methods

Understanding RNA interference.

The diffusion and transport of siRNAs are described in the next chapter, and the kinetics of siRNA in the cell are described in detail here. As endocytosis occurs, siRNA bound to vehicle (siRNA-vehicle) in the extracellular space (ECS) diminishes and endosomal siRNA-vehicle within the cell increases. The endocytosis rate is proportional to the amount of siRNA-vehicle entering the ECS (assumed constant for this model). Endosome bursts which releases the siRNA-vehicle occurs at first order rate depending on the concentration of the endosome in the cell. The concentration of siRNA-vehicle in the cytosol, or intracellular space (ICS) increases at the same rate the endosome bursts, but decreases as the siRNA dissociates with the delivery vehicle to form unbound siRNA. This dissociation is reversible but the dissociation reaction is favorable. The amount of free siRNA in the ICS (siRNA-ICS) increases. Free siRNA degrades according to first order kinetics. To participate in the gene silencing mechanism, intracellular siRNA needs to be incorporated in the RNA-induced silencing complex (RISC), and the binding of siRNA to RISC is reversible. siRNA binds to RISC with high affinity and low dissociation rate. RISC is contitutively expressed in high
concentrations in the cell, since RISC participates in the endogenous regulation of mRNA by microRNA. The RISC-siRNA complex identifies, binds, and cleaves targeted mRNA with high affinity. The siRNA-RISC oscillates in concentration because it binds to mRNA, degrades the mRNA, unbinds, and repeats. The number of targeted mRNA encoding for the NMDA receptors is not known, but the range of mRNA copies for different proteins in the cell ranges from single digits for rarely used proteins to thousands for house-keeping proteins or proteins expressed due to the specialization of the cell. As the siRNA-RISC complex binds to the mRNA, the mRNA concentration decreases proportionally with second order kinetics dependent on the amount siRNA-RISC and the amount of mRNA. The natural rate of production of NMDA receptors is a function of the translation of the existing pool of mRNA. NMDA receptors also has a natural turnover rate as they are degraded. In the absense of RNA interference, the amount of NMDA receptors in the cell reaches a steady state, where the expression rate equals the degradation rate. For the gene silencing of proteins with long half-lives, the response time characterizing the degradation of mRNA to the decrease in protein level is slow. If the targeted protein has a short half-life, the response time is short since the decrease in protein level will soon follow the degradation of mRNA.

**Desired downregulation**

Experimental data shows that a decrease in protein level of about 70-90% in rats through intrathecal delivery is proven effective in reducing chronic pain.

**Mathematical model.**

A mathematical model using a system of differential equations (ODE) was created similar to an example by the Bartlett and Davis. The model was generated in MATLAB. The mathematical model includes ten partial differential equations and was solved with MATLAB’s semi-implicit ODE integrator, ODE15s.

**siRNA delivery using endocytosis inhibitor**

This case-study was implemented in a commercial software FLUENT using our RNA interference kinetics in MATLAB as a user-input function. The target zone of our therapy is the dorsal horns of the spinal cord with a very high expression of NMDA receptors. Before reaching the dorsal horns, the untargeted cells will also endocytose siRNA along its diffusion path, which decreases significantly the bioavailability of siRNA at the target zones. It has been shown that an antibody of clathrin can inhibit clathrin-mediated endocytosis. Therefore, we design an

![Figure 22. Schematic of RNA interference in a single cell.](image)
infusion setting where a pretreatment of the spinal cord with endocytosis inhibitors is designed to decrease siRNA endocytosis only in its earlier diffusion pathway, but endocytosis is allowed to occur in the target zones.

Results

Ten differential equations describing the RNA interference mechanism is shown in Equation 7 to Equation 16.

\[ \frac{\partial C_{\text{DsiRNA-ECS}}}{\partial t} = R_{\text{macroscopic}} - k_{\text{endocytosis}} \times C_{\text{DsiRNA-ECS}} \]  

Equation 7

Equation 7 above represents the change in concentration of siRNA-vehicle complex in the ECS with respect to a change in time. The rate of complex fed into the ECS from the CSF and the rate of endocytosis are included in this description. This is described as an irreversible process.

Once the complex is encapsulated through endocytosis in an endosome the contents must somehow be released into the cytoplasm (intracellular space). The cellular process to do this is called endosomal burst [3]. Equation 8 below describes the change in siRNA-vehicle in the endosomes produced by the cell’s endocytosis with respect to a change in time.

\[ \frac{\partial C_{\text{endo-DsiRNA}}}{\partial t} = k_{\text{endocytosis}} \times (C_{\text{DsiRNA-ECS}} - C_{\text{DsiRNA-ICS}}) - k_{\text{endoburst}} \times C_{\text{endo-DsiRNA}} \]  

Equation 8

The rate of endocytosis and rate of the endosome bursting in the intracellular space (ICS) which are both irreversible processes affect Equation (2).

Endosomal Burst

\[ \frac{\partial C_{\text{DsiRNA-ICS}}}{\partial t} = k_{\text{endoburst}} \times C_{\text{endo-DsiRNA}} - k_{\text{siRNAseparationP}} \times C_{\text{DsiRNA-ICS}} + k_{\text{siRNAseparationB}} \times C_{\text{siRNA-ICS}} \times C_{D-ICS} \]  

Equation 9
Unpackaging of siRNA

\[
\frac{dc_{D-ICS}}{dt} = k_{siRNAseperationF} \times c_{DSiRNA-ICS} - k_{siRNAseperationB} \times c_{SiRNA-ICS} \times c_{D-ICS}
\]

\[
\frac{dc_{SiRNA-ICS}}{dt} = k_{siRNAseperationF} \times c_{DSiRNA-ICS} - k_{siRNAseperationB} \times c_{SiRNA-ICS} \times c_{D-ICS} - k_{RISCactivationF} \times c_{SiRNA-ICS} \times c_{RISC} + k_{RISCactivationB} \times c_{RISCsiRNA} - k_{siRNAtturnover} \times c_{SiRNA-ICS}
\]

RISC Activation

\[
\frac{dc_{RISC}}{dt} = R_{RISCproduction} - k_{RISCactivationF} \times c_{SiRNA-ICS} \times c_{RISC} + k_{RISCactivationB} \times c_{RISCsiRNA} - k_{RISCturnover} \times c_{RISC}
\]

\[
\frac{dc_{RISCsiRNA}}{dt} = k_{RISCactivationF} \times c_{SiRNA-ICS} \times c_{RISC} - k_{RISCactivationB} \times c_{RISCsiRNA} - k_{mRNAbindingF} \times c_{RISCsiRNA} \times c_{mRNA} + k_{mRNAbindingB} \times c_{mRNA-RISCsiRNA}
\]

mRNA Binding and Degradation

\[
\frac{dc_{mRNA}}{dt} = R_{mRNAproduction} - k_{mRNAbindingF} \times c_{RISCsiRNA} \times c_{mRNA} + k_{mRNAbindingB} \times c_{mRNA-RISCsiRNA} - R_{mRNAtturnover}
\]

\[
\frac{dc_{mRNA-RISCsiRNA}}{dt} = k_{mRNAbindingF} \times c_{RISCsiRNA} \times c_{mRNA} - k_{mRNAbindingB} \times c_{mRNA-RISCsiRNA} - k_{mRNAdegradation} \times c_{mRNA-RISCsiRNA}
\]

Protein Reduction

\[
\frac{dc_{protein}}{dt} = k_{proteinProduction} \times c_{mRNA} - R_{proteinTurnover}
\]

Where C is the concentration of each species. The values of all kinetic rates, \( k_i \), are shown in table Table 8. Max is the number of cells.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
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<td>max</td>
<td>Maximum # of cells (#)</td>
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</tr>
<tr>
<td>P</td>
<td>Effective fraction of dose available to target cells</td>
<td>1e-3</td>
</tr>
<tr>
<td>R</td>
<td>Total available amount of RISC protein complexes</td>
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<td>Ve</td>
<td>Extracellular volume (L)</td>
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<td>Vi</td>
<td>Intracellular volume (L)</td>
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<td>Vp</td>
<td>Plasma volume, mouse (L)</td>
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<td>k1</td>
<td>Complex binding to blood components (h_1)</td>
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<td>k2</td>
<td>Complex dissociation from blood components (h_1)</td>
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<td>Cleavage of target mRNA by activated RISC complex (h_1)</td>
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<td>Endosomal siRNA degradation (h_1)</td>
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<td>Intracellular siRNA degradation (h_1)</td>
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<td>Plasma complex degradation (h_1)</td>
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<td>Endosomal escape for siRNA (h_1)</td>
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<td>k14</td>
<td>Endosomal escape for complex (h_1)</td>
<td>1e-2</td>
</tr>
<tr>
<td>k15</td>
<td>Formation of target mRNA (# L_1h_1)</td>
<td>5.2e13</td>
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<td>Formation of target protein (h_1)</td>
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<td>k17</td>
<td>Formation of activated RISC complex (L #_1h_1)</td>
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<tr>
<td>k20</td>
<td>Internalization (h_1)</td>
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<td>k21</td>
<td>Transport from plasma to extracellular fluid (h_1)</td>
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<td>Cytosolic complex unpackaging (h_1)</td>
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<td>dissociation of siRNA and vehicle PEI in CSF (1/h)</td>
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<td>k_{anti-clath}</td>
<td>binding antibody to clathrin for endocytosis(1/h)</td>
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</table>
The integration of these partial differential equations yields time-dependent trajectories of all biochemical species for the RNA interference pathways. Furthermore, the protein downregulation was shown for four different siRNA initial concentrations to demonstrate the response of this kinetic system to different input concentrations, as shown in Figure 23.

The 2e23 line corresponding to 10nM siRNA, the 4e23 line to 25 nM, the 5e23 line to 50 nM, and the 6e23 line to 100nM. These concentrations achieved 20%, 45%, 60%, and 75% of downregulation, respectively.

**Case-Study: Co-infusion of endocytosis-inhibitor with siRNA**

siRNA is typically chosen to be delivered with a vehicle molecule. This is because the siRNA degrades in the CSF due to natural nucleases in the fluid.(64) A common vehicle used is polyethyleneimine (PEI) which is a cationic polymer that protects the siRNA by neutralizing the phosphates on the siRNA with its nitrogen groups.(65) We intrathecally delivered siRNA bound to PEI and simulated a 90 second injection.

High doses of siRNA are required for producing a satisfactory effect, due to the non-specific endocytosis of siRNA molecules by targeted and untargeted cells. A clathrin-antibody was found to effectively inhibit clathrin-mediated endocytosis.(66) In the simulation, the concentration of clathrin antibody was held constant in the CSF. A mass-transfer boundary condition at the spinal cord surface allows for the diffusion of the antibody into the spinal cord. As the diffusion occurs, the antibodies bind to clathrin molecules in cells. These molecules are responsible for endocytosis of the siRNA.(67) When bound by the antibodies, clathrin is unable to aid in endocytosis. The idea with co-infusion of siRNA therapies and a decoy drug is to improve the efficacy of siRNA by decreasing the amount of non-specific intracellular uptake outside of the dorsal horns. Therefore, the timing of clathrin antibody infusion is crucial since the inhibition of endocytosis in targeted cells will abolish the effect of siRNA. Therefore, a pre-infusion of clathrin before siRNA infusion is desirable, and the optimal time interval can be computed using this computational design. When the siRNA is infused later it bypasses the cells in the spinal cord that have already been affected by the antibodies. More siRNA can reach the dorsal horns within the spinal cord. Figure 24 shows the distribution of antibody after infusion.
Discussion.

The construction of cellular kinetic models describing RNA interference has successfully described experimental data in vitro, see Figure 25. Downregulation of target protein as quantified by luciferase signal was obtained for four different siRNA concentrations. These protein concentrations were well matched by a mathematical model using differential equations to describe the mechanisms of gene silencing similar to our model. The prediction of RNA interference effect in vitro has been done.

However, understanding the input-put relationship in vitro does not provide optimal dosing criteria for infusion trials for animals or humans. The computational design methodology coupling single-cell kinetics and macroscopic transport properties within the framework of patient-specific reconstruction can predict a range of effective infusion concentrations for experimental testing. This integration is shown in Figure 26.

The commercial software, FLUENT, has several shortcomings when it comes to implemented complex cellular kinetics, since this software was designed to compute bulk reactions. One of the shortcomings was its inability to generate immobile chemical species, such as receptors. Therefore, in the following chapter, the integration of the kinetic model with patient-specific geometry of the spinal cord will be performed in our own code.
Figure 25. Figures by Barlett and Davis (68) shows that the effect of gene silencing as quantified by luciferase signals is well-predicted by a mathematical model using a system of differential equations.

Conclusion
We have generated a novel approach for bridging the gap between useful in vitro data of gene silencing efficacy and designing optimal infusion therapies. A single-cell mathematical model describing RNA interference was created and integrated within a organ-wide framework of the spinal cord.

Figure 26. The combination of kinetic models with patient-specific computations yields effective concentration range for intrathecal infusions.
Chapter VI. Medical Image-based Systematic Design of Human Gene Silencing Therapies

Abstract

**Background.** Gene silencing therapies have succeeded in controlling expression levels of a desired gene in animal models. By infusing short-interfering RNAs (siRNA), these molecules target particular messenger RNA (mRNA) in the cells through sequence-specific binding, suppressing translation. These therapies hold great promise for treating numerous disorders of the central nervous system (CNS) including novel approaches to chronic pain management. While novel siRNA targets are being discovered rapidly, difficulties in siRNA delivery such as anatomical accessibility of the target tissue, slow diffusion and non-specific uptake make achieving a precise degree of protein downregulation nearly impossible. We propose to design optimal infusions integrating medical imaging with systems engineering principles.

**Method.** The design of a novel pain therapy to suppress the expression of pain-transducing NMDA receptors in the subject's spinal cord was performed through mathematical modeling. The subject's spinal cord was reconstructed from magnetic resonance (MR) images. Biotransport equations were coupled with intracellular siRNA kinetics.

**Results.** By coupling siRNA kinetics and organ-wide siRNA biotransport, the biodistribution and action of siRNAs infused intrathecally were predicted. Results show percent NMDA receptor downregulation over time with different infusion concentrations of siRNA.

**Conclusion.** The accurate prediction of dose-response relationship and the computation of optimal infusion parameters are expected to accelerate clinical implementations of gene silencing therapies.
Introduction

The translation of bench-top success in gene silencing therapeutics towards clinical implementation is traditionally accomplished through animal testing from small rodents to primates. RNA interference targeting NMDA receptors in the spinal cord through intrathecal delivery has shown great promise for the novel treatments for chronic pain management. Although promising results have been obtained in animal trials, animal testing does not provide optimal dosing guidelines for clinical application in humans. The anatomy of CNS for human differs drastically from rodents, and the larger size of cerebrospinal fluid space, spinal cord and brain requires siRNA diffusion without being degraded by nucleases. The accurate scaling of dosage is required to accomplish similar distribution and effect of gene silencing in humans. Developing infusion therapies based solely on animal testing has a few disadvantages. 1) The difference between animal and human CNS physiology is great. 2) Incorrect scaling leads to ineffective dosing or overdose. 3) animal experiments are time consuming and not cost-effective. 4) Animal experiments do not reflect patient-to-patient differences in anatomy, drug biotransport, and metabolic rates, and they cannot address the important issue of subject-specific infusion therapies.

The infusion of therapeutics into the human CNS is particularly challenging due to the blood brain barrier, which prevents macromolecules from leaving the cerebrovasculature. To reach the brain or spinal cord tissue effectively, therapeutics can be instead infused into the cerebrospinal fluid (CSF), which surrounds the entire CNS. After infusion, the biodistribution and reactions of these therapeutic molecules are unknown. What is the distribution of these molecules along the spine? What percentage of the infused molecules reached the target? Can we quantify the biochemical interaction of siRNA with the tissue, such as binding, internalization, or enzymatic activation of other intracellular proteins? We provide quantitative answers to these open questions using medical image-based computational fluid dynamics, termed miCFD. Following the prediction of optimal dose range, the experimental validation through animal experiments is still needed. However, this methodology can streamline experimental design by generating a infusion dose-range that is likely effective, thus reducing experimental cost and time devoted to deriving dose-response relationship in repetitive animal trials.
Figure 27. (A) Image reconstruction of the subject’s CNS. (B) Image of the human spinal cord (top frame) and the reconstructed model with different zones (bottom frame). The target zones are the dorsal horns, densely populated with NMDA receptors.

Methodology

1.1. Three dimensional reconstruction of the human CNS

A patient-specific model was reconstructed from magnetic resonance (MR) images of the patient’s CNS through a process termed image reconstruction (Figure 27). This patient-specific model was converted into an unstructured computational mesh as described in Somayaji et al.

1.2. Functional regions in the spinal cord

The human spinal cord is composed of the grey and white matter. Within the grey matter, our target regions are within the dorsal horns where the NMDA receptors are densely populated (Figure 27). Neurons within these target regions specialize in pain signal transduction through these receptors. Neurons outside of the dorsal horns have little or no target receptors. Therefore, siRNA molecules have no effect in neurons outside of the target regions due to the lack of NMDA receptor-encoding mRNA. The bioreactivities of siRNA molecules differ in different functional regions in the spinal cord. The application of transport and reaction equations allows the computation of infusion, biodistribution, and reactions of siRNA within different cell types.

1.3. Gene silencing therapies for chronic pain

The goal of this therapy is to silence 70% of the pain-transducing NMDA receptors in the subject’s spinal cord to suppress chronically heightened pain. We estimate the siRNA infusion concentration that would
achieve this precise demand. NMDA receptor down-regulation in the spinal cord is computed for four different siRNA concentrations in the CSF, simulating continuous spinal infusions. The continuity (2) and species transport (3) equations compute the biotransport of siRNA.

The cells in grey and white matter of the spinal cord actively uptake siRNA molecules in the extracellular space. In untargeted cells lacking NMDA receptors and their corresponding mRNA, siRNA molecules accumulate and are merely degraded. Inside the target cells, the activation of the gene silencing cascades is initiated by the binding of siRNA to an intracellular protein termed RNA-induced-silencing-complex (RISC). The activated siRNA binds to the NMDA receptor-encoding mRNA with high affinity and specificity. The RISC-siRNA cleaves the bound mRNA, and the translation of the receptor is suppressed for as long as an effective siRNA concentration is present in the cell. These biochemical reactions are computed for extracellular siRNA (4), intracellular siRNA (5), RISC (6), activated RISC-siRNA complex (7), bound RISC-siRNA-mRNA (8), target mRNA (9), and NMDA receptors (10). These equations are simplified from the mathematical model presented in the last chapter.

\[
\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho v) = 0 \tag{2}
\]

\[
\frac{\partial}{\partial t} (\rho Y_i) + \nabla \cdot (\rho v Y_i) = -\nabla \cdot J_i + S_i \tag{3}
\]

\[
\frac{\partial C_{\text{siRNAex}}}{\partial t} = -k_1 \cdot C_{\text{siRNAex}} - k_2 \cdot C_{\text{siRNAex}} \tag{4}
\]

\[
\frac{\partial C_{\text{siRNAin}}}{\partial t} = +k_1 \cdot C_{\text{siRNAex}} - k_3 \cdot C_{\text{siRNAin}} \cdot C_{\text{RISC}} \tag{5}
\]

\[
\frac{\partial C_{\text{RISC}}}{\partial t} = P_{\text{RISC}} - k_3 \cdot C_{\text{siRNAin}} \cdot C_{\text{RISC}} + k_6 \cdot C_{\text{RISC-siRNA}} - k_{10} \cdot C_{\text{RISC}} \tag{6}
\]

\[
\frac{\partial C_{\text{RISC-siRNA}}}{\partial t} = k_3 \cdot C_{\text{siRNAin}} \cdot C_{\text{RISC}} - k_4 \cdot C_{\text{RISC-siRNA}} \cdot C_{\text{mRNA}} + k_5 \cdot C_{\text{RISC-siRNA-mRNA}} - k_6 \tag{7}
\]

\[
\frac{\partial C_{\text{RISC-siRNA-mRNA}}}{\partial t} = k_4 \cdot C_{\text{RISC-siRNA}} \cdot C_{\text{mRNA}} - k_5 \cdot C_{\text{RISC-siRNA-mRNA}} \tag{8}
\]
In these equations, \( C \) is species concentration; \( P_{RISC} = k_4 \cdot C_{RISC-siRNA} \cdot C_{mRNA} - k_7 \cdot C_{mRNA} - k_8 \cdot C_{mRNA} \) (9) \[ \frac{\partial C_{mRNA}}{\partial t} = P_{mRNA} - k_4 \cdot C_{RISC-siRNA} \cdot C_{mRNA} - k_7 \cdot C_{mRNA} - k_8 \cdot C_{mRNA} \]

\[ \frac{\partial C_{receptor}}{\partial t} = k_8 \cdot C_{mRNA} - k_9 \cdot C_{receptor} \] (10)

In these equations, \( C \) is species concentration; \( P_{RISC} = 5 \cdot 10^8 \) is the RISC production rate, and \( P_{mRNA} = 5 \cdot 10^9 \) is the mRNA production rate. Reaction rate constants used were derived from experimental data by Bartlett and Davis.(68)

Results and Discussion

Prior to siRNA treatment, the NMDA receptors and their encoding mRNA in the target cells maintain constant levels. These steady state levels are responsible for the stable receptor expression in these cells. The number of 'cells' per \( \text{cm}^3 \) is estimated based on the extracellular volume fraction and the average cell volume. This allows us to estimate the number of molecules per cell from computed concentrations.

At steady state, there are 55,543 NMDA receptors and 900 receptor-encoding mRNA per cell. The quantities of mRNA and protein are within reported range for mammalian genes.(72) As expected, the amount of receptors per cell is much greater than its encoding mRNA,(72) due to translational amplification. Each mRNA transcript is used for translation multiple times before its natural degradation.

After the start of a continuous siRNA infusion, the system in steady state experiences a dynamic transition. siRNA molecules diffuse into the tissue from the spinal cord surface. Cellular uptake of siRNA molecules occurs simultaneously. A small amount of siRNA molecules reach the dorsal horns and initiate gene silencing. siRNA concentration inside dorsal horn cells is several orders of magnitude smaller than the infusion concentration due to the diffusion resistance and non-specific cellular uptake.

Intracellular siRNA in a cross section of the spinal cord is shown for four hours of continuous infusion at the concentration of \( 10^{-6} \) M (top panel of Figure 28). The receptor down-regulation in the dorsal horns is shown in the bottom panel (Figure 28).
Figure 28. Intracellular siRNA (top) and NMDA receptors in the dorsal horns (bottom) in a cross section of the spinal cord over four hours of continuous siRNA infusion at $10^{-6}$ M. (Top panel) siRNA molecules are uptaken by cells during their diffusion. Slow diffusion and non-specific uptake of siRNA are principle barriers for effective delivery of siRNA into a targeted region in the CNS. (Bottom panel) gene silencing of pain-transducing NMDA receptors induced by intracellular siRNA molecules in the dorsal horns.

Four different infusion concentrations were compared for gene silencing efficacy using this patient-specific model. NMDA receptor down-regulation for continuous siRNA infusions into the CSF over 9 hours is shown in Figure 29. After gene silencing, the target receptors were 92.9%, 85.9% 57.6% and 29.4% of the steady state level for siRNA infusion concentrations of $10^{-7}$M, $2 \cdot 10^{-7}$M, $6 \cdot 10^{-7}$M and $10^{-6}$M, respectively. The therapy goal of 70% NMDA receptor suppression was met using an injectate with $10^{-6}$M siRNA concentration.
Figure 29. Pain-transducing NMDA receptor down-regulation for 9 hours of continuous siRNA infusion. The infusion concentration of $10^{-6}$M induces 70.6% of receptor suppression, meeting the therapy goal. The optimal infusion concentration is determined.

**Conclusions**

Controlling the expression of disease-related genes in the CNS using gene silencing therapies is the next generation of CNS treatments. However, delivering an exact concentration of siRNA molecules to a targeted region in the CNS presents multiple challenges. CNS therapy design also poses stringent requirements. For example, a precise percent of protein down-regulation may be desired in the target region, while maintaining unaltered protein levels elsewhere. In many cases, it is not desirable to completely suppress the expression of a target gene. The use of sub-optimal infusion parameters may cause either no effect on the gene expression or the complete suppression of a gene, leading to unwanted outcomes.

The traditional trial and error animal infusion experiments do not provide quantitative answers for the optimal dosing of a human subject. This chapter addresses the challenge of optimal human dosing by integrating medical imaging with systems engineering principles. siRNA biochemical kinetics coupled with biotransport are fused with subject-specific anatomy to estimate drug action in vivo. The systematic design of human gene silencing therapies can generate optimal infusion parameters to precisely suppress protein expression levels as desired. Future direction includes the coupling of this patient-specific model with spatially distributed kinetic inversion technique for the determination of unknown siRNA reaction parameters.
Chapter VII. Activation of a neuroprotective transcription factor and aquaporin-4 upregulation in brain astrocytes.

Abstract.

**Background.** Aquaporin-4 (AQP4) is a water channel in the brain. AQP4 plays an important role in water management in the brain, and its expression levels affects the outcomes of edema, hydrocephalus, and traumatic brain injury. AQP4 shows dynamic temporal trajectories and complex spatial distribution patterns in the brain after neurological injury. In hydrocephalus, AQP4-null mice have more severe neurological deficit, worse intracranial pressure, and higher mortality rate compared to AQP4 expressing mice. We investigated molecular strategies to upregulate AQP4 water channels for developing novel pharmacological treatment options for hydrocephalus. We also tested the activation of Nrf2, a transcription factor involved in the activation of genes containing the anti-oxidant response element including aqp4 gene.

**Methods.** We used primary astrocyte cell culture to test the effect of sulforaphane on AQP4 expression. The purity of the astrocyte culture was confirmed with astrocytic marker GFAP. For the first time in our lab, an immunocytochemistry protocol was established to visualize cellular proteins with antibodies. Protocols were also established to passage and maintain an astrocyte culture. After exposure to sulforaphane, cells were fixed and labeled with anti-Nrf2 antibodies. The expression levels of AQP4 was quantified using western blot. Bradford assay was performed to quantify protein concentrations in cell lysates, followed by gel electrophoresis and chemiluminescence imaging. The intensities of AQP4 bands were quantified with ImageJ.

**Results.** Sulforaphane upregulated AQP4 in primary astrocyte cells. The translocation of Nrf2 from the cytoplasm into the nucleus after sulforaphane stimulation was observed by immunocytochemistry.

**Conclusions.** Nrf2 is known to activate Phase-II detoxifying genes containing the antioxidant-response element and offers cyto-protection. Sulforaphane, an Nrf2 inducer as shown by our experiments and others, has no known side effects. Based on the upregulation of AQP4 water channels in astrocytes by sulforaphane, we conclude that sulforaphane is a promising candidate for novel pharmacological intervention therapies for hydrocephalus.
Introduction

Hydrocephalus (HC) afflicts nearly 1 in 500 new borns. In HC, excess cerebral spinal fluid (CSF) is accumulated in the ventricles of the brain, leading to high intracranial pressure. The causes of HC could be the over-production of CSF by the choroid plexus or reduced absorption into the arachnoid villi. HC is normally classified into two subtypes: (1) communicative HC, in which a blockage in the absorption pathway does not exist; and (2) non-communicative HC, in which the drainage of CSF is prevented by a physical blockage in the pathway. The only current treatment for HC is an invasive surgery to place a shunt in the skull to drain excess CSF into the abdomen. Shunt placement surgeries have high failure and infection rates. Thus, we aim to identify a novel pharmacological treatment option to noninvasive enhance the clearance of CSF from the brain.

Aquaporin-4 (AQP4) channels are the most ubiquitous water channels in the central nervous system. They are transmembrane proteins which serve as bidirectional conduits with high selectivity for water transport along osmotic gradients.(73-74) AQP4 channels are highly concentrated in astrocytic endfeet,(75) especially near the glial limitans facing the cortical and ventricular surfaces, as well as the basolateral membranes of the ventricular ependyma. (76) AQP4 - together with potassium channels Kir4.1 - also control osmotic balance,(77) and mediate astrocytic re-uptake of water at gap junctions.(77) A growing number of studies is beginning to elucidate the central role of astrocytes in brain water management via AQP4 channels. The localization of AQP4 channels at the blood-brain and the blood-CSF barrier is consistent with their potential role in regulating the transport of water in and out of the brain parenchyma.(75, 78-79)

AQP4 expression in astrocytes is not only related to water conduction across cellular membranes, but also directly impact brain water permeability. Gunnarson,(81) Solenov,(73) and Nicchia(82) have discovered that manipulating the expression levels of AQP4 in astrocytes controls water permeability through cellular...
membranes. The water permeability of normal astrocytes expressing AQP4 is 3-fold\(^{(81)}\) to 7-fold\(^{(73)}\) higher than astrocytes without AQP4. These studies show that water permeability through astrocytic membranes is controlled by the expression levels of AQP4. On the organ-wide level, Badaut showed that AQP4 gene silencing in rat decreased brain water permeability by 50\% with diffusion tensor imaging (DTI).\(^{(83)}\) AQP4-deficient mice showed a greater severity in hydrocephalus progression.\(^{(84)}\) Even though it is not verified whether astrocytes can transport water across the brain parenchyma acting as cellular conduits, the experimental evidence points to AQP4’s role in bulk fluid exchange in the brain. The upregulation or downregulation of AQP4 by pharmacological agents are promising strategies to treat neurological disorders of brain water regulation.

We hypothesize that AQP4 upregulation would assist in the clearance of excess CSF in hydrocephalus. Using primary astrocyte cultures, we investigated the expression of AQP4 after sulforaphane (SUL) exposure. The effect of SUL on Nrf2, a putative transcription factor of the aqp4 gene, was investigated by immunocytochemistry. We have established for the first time in this laboratory cell culture as well as immunocytochemistry techniques.

**Sulforaphane induced upregulation of AQP4**

SUL is a natural isothiocyanate present in abundance in broccoli and other cruciferous vegetables. As an antioxidant, it acts to prevent free radical formation and activates the phase-II detoxifying genes. Zhao showed that SUL administration in rats after TBI enhanced their performance in Morris water maze test.\(^{(85)}\) In addition, Zhao studied the connection between SUL and Nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 or Nrf2. SUL is an inducer of the antioxidant-responsive element (ARE).\(^{(85)}\) It does this by activating Nrf2, increasing the expression of the ARE genes by binding to the promoter region of these target genes. Danilova et al showed that SUL induces upregulation of NAD(P)H Quinone oxidoreductase 1 (NQO1) mRNA in rat astrocytes. They concluded that sulforaphane stimulates the Nrf2 pathway of antioxidant gene expression in astrocytes and protects them from cell death in an *in vitro* model of ischemia.\(^{(86)}\) Kang et al investigated the activation of the ARE by tert-Butylhydroquinone (t-BHQ) and the involvement of Nrf2 using rat hepatoma cells.\(^{(87)}\) They determined that Nrf2 binds covalently to actin, a
microfilament, in the cytoplasm and that actin may also participate in the process of nuclear translocation. PI3K mediates the dynamic reorganization of actin.

**Function and mechanisms of sulforaphane**

The Kelch-like-ECH-associated protein 1 (Keap1) is a cytoplasmic repressor of Nrf2 that inhibits its ability to translocate to the nucleus. (80) SUL directly interacts with Keap1 and releases Nrf2, which is now free to translocate to the nucleus. In the nucleus, Nrf2 associates with small MAF proteins (the term MAF is derived from usculoaponeurotic-fibrosarcoma virus), forming a heterodimer that binds to ARE to stimulate gene expression. Danilov points out the induction of the phase-II gene as a response to stress involves the PI3K and perhaps PKC pathways, and they could activate Nrf2 through phosphorylation. (86) On the other hand, Wang reports that Akt (protein kinase B) may be activated by PI3K and then phosphorylate Nrf2. (88)

In this chapter, the activation of Nrf2 by SUL was tested by immunocytochemistry in primary astrocyte cultures. Subsequently, the upregulation of AQP4 by SUL was quantified by western blotting.

**Methodology**

**Cell culture.** Primary rat cortical astrocytes were purchased from Lonza. Cells were grown in astrocyte growth medium containing 20% serum and 1% antibiotics. Medium was changed twice a week, and cells were passaged once they reach 100% confluency.

**Sulforaphane exposure.** 200µL of 5µM SUL (Sigma Aldrich) was added in our astrocyte cultures for the indicated durations. Control cells received medium without SUL.

**Immunocytochemistry.** The purity of our culture was confirmed by labeling the glial fibrillary acidic protein (GFAP) astrocyte marker using immunocytochemistry. Cells were fixed in 4% paraformaldehyde for about 20 minutes, permeabilized with acetone for 3 minutes, and blocked with bovine serum albumin (BSA) for about 40 minutes. After rinsing with PBS, cells were then incubated with GFAP primary antibody conjugated to streptavidin for 1 hour and the streptavidin was recognized by biotin-conjugated TRITC. For labeling Nrf2, mouse anti-Nrf2 primary antibody (Abcam) and goat anti-mouse secondary antibody tagged with Alexa 488
(Jackson Immuno Research) were used. Lastly, cells were labeled with DAPI and visualized with Zeiss fluorescence microscope at 40x magnification.

**Western blotting.** Cells were incubated with SUL and AQP4 levels were quantified. 9 and 18 hours after continuous SUL (or control medium) exposure, cells were disrupted, cell lysates were prepared on ice with protease inhibitors. Bradford assay was performed to ensure equal loading of proteins in all samples. Protein samples were heated at 95 degrees for 5 minutes and loaded into the wells in gels. The western blot data was analyzed with densitometry in ImageJ.

**Results.**

**Astrocyte culture and expression of GFAP.** The morphology of astrocyte culture was monitored by light microscopy, see Figure 31. After arrival and replating, cells show processes. The culture was a mixture of stellated and polygonal cells. After a few passages, most cells were polygonal in shape. We verified the purity of astrocytes by the GFAP antibody, as shown in Figure 33.

![Figure 31. After replating, astrocytes form processes.](image1)

![Figure 32. Four days after replating, astrocytes show extended processes.](image2)
**Nrf2 activation by SUL in astrocytes.** We performed immunofluorescence to confirm Nrf2 translocation by SUL. After 90 minutes, cells were fixed with 4% paraformaldehyde and permeabilized with acetone. Immunocytochemistry shows that Nrf2 is found in the peri-nuclear region and cytoplasm in its inactive state with a weak basal nuclear staining as shown in Figure 34, consistent with prior observations. After SUL activation, high Nrf2 immunoreactivity was observed inside the nucleus, indicating a translocation of Nrf2 from the cytoplasm to the nucleus as shown in Figure 35. Our Nrf2 translocation results confirmed earlier findings. The osmolarity of the SUL solution and control medium was measured using vapor-pressure osmometer. The osmolarity of the SUL medium and DMSO-control medium were 348.67 mOsmo/kg and 332 mOsmo/kg, respectively. The osmolarity of the regular culture media was 295 mOsmo/kg. The SUL and DMSO-control media were slightly hyperosmolar compared to regular medium.
In Figure 35, AQP4 shows punctate staining on the membrane. The dotted pattern could be related to the expression of orthogonal arrays on the membrane. It has been shown that AQP4 forms large arrays which have functions of adhesion and increased water transport.

**AQP4 trafficking in astrocytes.** We labeled AQP4 using mouse anti-AQP4 primary antibody (Abcam) and goat anti-mouse secondary antibody tagged with Alexa488 (Jackson ImmunoResearch). Figure 36 shows AQP4 localization in the endfeet and filled vesicles in the cell body of a single astrocyte. Intracellular AQP4 in vesicles indicates the trafficking of AQP4 between cell body and the endfoot processes.

**AQP4 upregulation by SUL.** Western blot (WB) was performed to quantify the upregulation of AQP4 by 5µM of SUL. 9 and 18 hours after continuous SUL (or control medium) exposure, cells were disrupted, cell
lysates were prepared and Bradford assay was performed to ensure equal loading of proteins in all samples. The results show that AQP4 expression was 1.48 and 1.68 fold higher after 9 and 18 hours of SUL exposure as shown in Figure 36. Our preliminary data confirmed that SUL induces upregulation of AQP4.

Discussion

AQP4 expression can be activated by hyperosmolarity. Our SUL-medium solution has the osmolarity of 348mOsmo/kg, which was significantly higher than the control medium. In the future, we have to eliminate the possibility that AQP4 upregulation observed was not due to the hyperosmolarity of the SUL solution, but due to SUL exposure itself. SUL was dissolved in DMSO before its dilution in culture media to the desired concentration. The effect of DMSO on AQP4 should be examined.

In some cells, Nrf2 was observed in the peri-nuclear region, while in others a scattered expression was observed throughout the cell. However, a filamentous staining pattern was consistently observed in all Nrf2 staining, in line with the fact that Nrf2 is actin-bound in the cytoplasm. Kang states that Nrf2 would be found bound to actin throughout the cell. Nrf2 translocation should be confirmed by cellular fractionation using western blotting.

Lastly, performing three color staining using our Zeiss fluorescence microscope seemed to reveal the problem of bleed-through. This problem was observed using the combination of Alexa488 and TRITC, as well as Alexa488 with Alexa594. If the correct excitation wavelengths were used, Alexa488 and Alexa594 should provide emission spectra with reasonable optical separation. However, this problem is likely due to the inappropriate selection of the excitation and emission filters. The selection of fluorophores with excitation spectra appropriate for our filters or the installation of correct excitation and emission filters could be the solution to this issue.

Conclusion.
Sulforaphane is a natural substance found in cruciferus vegetables. The beneficial effects of SUL in the CNS are mainly due to the activation of a neuro-protective transcription factor, Nrf2. Since AQP4 contains putative AREs in the promoter region, Nrf2 is suspected to modulate SUL-induced AQP4 upregulation. In this chapter, we have shown the activation of Nrf2 after SUL exposure, and the upregulation of AQP4. However, a causal effect of Nrf2 activation and AQP4 upregulation needs to be further demonstrated using inhibitory antibodies against Nrf2. Nevertheless, we have demonstrated that SUL is a potent activator of AQP4 expression in astrocytes, and it is a promising candidate for upregulating AQP4 in the brain in vivo.
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Appendix 1. Solving the Navier Stokes equation dynamically.

Abstract

**Background.** Navier Stokes equation describes the relationship between pressure and velocity, and is used to solve for the complete fluid and pressure fields when the boundary conditions of a domain is known. The Semi-Implicit Method of Pressure Linked Equations (SIMPLE) algorithm, proposed by Patankar in 1980, can be used to simplify the non linear Navier-Stokes equation into a system of linear algebraic equations.

**Methods.** In this section, the SIMPLE algorithm is programmed in MATLAB in order to solve the Navier-Stokes equation in a 2D domain with walls defined with no-slip boundary conditions. To solve for the velocity and pressure fields of a domain, sufficient boundary conditions are necessary such that degree of freedom criteria are met. The SIMPLE method is an iterative process; the velocity in the convection term and the pressure term are initially guessed, and the velocity field is solved based on the initial guess. This calculated value is then evaluated using the continuity equation for incompressible fluids and an updated pressure and velocity field is then calculated. The correctness of the MATLAB code is validated with simulations using FLUENT, a commercial software.

**Results.** MATLAB code and FLUENT generated comparable velocity flow fields in identical geometries, with laminar flow established within 0.5cm of a 5cm tube with an internal diameter of 0.5cm as predicted by the analytical equation for fully developed profiles.

**Conclusion.** The MATLAB implementation of SIMPLE algorithm for the iterative solution of Navier Stokes equation generates results comparable to the commercial solver FLUENT.
Introduction

The Navier-Stokes equation is a conservation balance of momentum describing fluid flow, see Equation 18. The Navier-Stokes equation is an application of Newton’s force conservation law, where the forces that act upon the transport of a fluid are described in terms of diffusion, convection and pressure gradient. The equation takes into account the continuity equation (Equation 17) of an incompressible fluid.

\[ \nabla \cdot \vec{u} = 0 \quad \text{Equation 17} \]

\[
\rho \frac{\partial \vec{u}}{\partial t} = -\nabla p - \rho \vec{u} \cdot \nabla \vec{u} + \mu \nabla \cdot \left( \nabla \vec{u} \right) \quad \text{Equation 18}
\]

Despite its immensely broad applications, the existence of the solution and smoothness to the Navier-Stokes equation are not proven, and there are no analytical solutions to the Navier-Stokes equation for flows in a spatially complex domain. Fluid mechanics in the realm of biological or biomedical engineering often involves studying flows in a complex anatomical domain, such as the dynamic blood flow in the cerebrovasculature or the cerebrospinal fluid pulsations in the subarachnoidal space in the brain. Therefore, numerical techniques to solve the Navier-Stokes equation are extremely useful for examining flows in biological systems.

Taking a closer look at the Navier-Stokes equation, it can be deduced that the difficulty in solving the Navier-Stokes equation arises from the non-linear convection term. Though employing non-linear numerical methods such as the Newton-Rhapson method may seem ideal, but these methods have proven to be incompatible with solving particularly large meshes. The SIMPLE algorithm can be used to linearize the momentum balance equation so that linear numerical methods can be used to handle solving fluid dynamics over a large distributed space.

The SIMPLE algorithm uses iterative methods to simplify the non-linear Navier-Stokes into a system of linear algebraic equations in order to solve the pressure and the velocity fields.

This appendix will cover three principle topics: Methodology of the SIMPLE algorithm, results in both the structured mesh, validation of code with third party, commercial computational fluid dynamics solver.

Methodology of the SIMPLE Algorithm

The SIMPLE algorithm is developed by Pantankar.(90) Using the finite volume methods, The SIMPLE method can be briefly explained using the following flowchart outlining the necessary steps.
In the case of a 2D spatial coordinate system the linear momentum equations are broken down into two equations for the $x$ and $y$ components of the velocity field.

\[
\nabla \cdot \vec{u} = 0
\]

\[
\rho \frac{\partial u}{\partial t} = -\nabla p - \rho \vec{u} \cdot \nabla \vec{u} + \mu \nabla \cdot (\nabla \vec{u})
\]

\[
\rho \frac{\partial v}{\partial t} = -\nabla p - \rho \vec{u} \cdot \nabla \vec{v} + \mu \nabla \cdot (\nabla \vec{v})
\]

Where $\rho$ and $\mu$ are density and viscosity of the bulk, $u$ and $v$ are the respective $x$ and $y$ components of the vector velocity field.

**2.1 Assume initial pressure and velocity field**
Assume initial values for the pressure field \( p^{k-1} \) and the velocity field components \( u^{k-1} \) and \( v^{k-1} \). Possible choices for the initial guesses include velocities equal to the inlet velocities and for pressures one can start with the outlet pressures.

In the unlikely scenario that the initial guesses are indeed the correct values then the algorithm will converge on the first iteration in this unlikely scenario. Otherwise, the algorithm will make a new guessed field.

2.2 Solve velocity field components \( u^* \) and \( v^* \)

The guessed pressure and velocity fields are replaced in the linear momentum equations.

\[
\rho \frac{\partial u}{\partial t} = -\nabla p^{k-1} - \rho u^{k-1} \cdot \nabla u^* + \mu \nabla \cdot (\nabla u^*) \quad \text{Equation 22}
\]

\[
\rho \frac{\partial v}{\partial t} = -\nabla p^{k-1} - \rho u^{k-1} \cdot \nabla v^* + \mu \nabla \cdot (\nabla v^*) \quad \text{Equation 23}
\]

The scalar values \( u' \) and \( v' \) are the solution variables of the velocity field. These values are related to the velocities field used for checking convergence by the relation

\[
\tilde{u} = \begin{pmatrix} u \\ v \end{pmatrix} = \begin{pmatrix} u^* \\ v^* \end{pmatrix} + \begin{pmatrix} u' \\ v' \end{pmatrix} \quad \text{Equation 24}
\]

Where \( u' \) and \( v' \) are the corrected values between the calculated value of a given iteration and the final converged solution where they are equivalent to zero. The discretized version of the linear momentum equation rearranged to solve for \( u^* \) and \( v^* \) in terms of \( p^{k-1} \) is shown below.

\[
\sum_{f=1}^{\text{# of faces}} p^{k-1} \cdot y_f = \sum_{f=1}^{\text{# of faces}} \left\{ \begin{array}{l}
\frac{\mu}{q_f} J_f' - \frac{1}{2} \rho \left( u^{k-1} y_0 - v^{k-1} x_0 \right) u_{0}^* \\
- \frac{\mu}{q_f} J_f' - \frac{1}{2} \rho \left( u^{k-1} y_0 - v^{k-1} x_0 \right) u_{0}^*
\end{array} \right\} \quad \text{Equation 25}
\]

2.3 Pressure correction formula

In earlier versions of the SIMPLE algorithm implemented in our lab, the calculation of \( p' \) was derived from the assumption that the pressure gradient across a given face is proportional to the calculated velocity at that same face. The proportionality constant involved only the hydraulic conductivity \( \kappa \) and the fluid viscosity \( \mu \). This assumption resulted in slow convergence of the continuity and of the \( y \)-component of the velocity fields in Cartesian grids. In this report, we propose a pressure correction formula that is consistent with the mathematical canon of the SIMPLE algorithm\(^3\). The derivation of the pressure correction term can be found in the appendix.

2.4 Update the pressure and velocity field

The pressure field can now be updated using the equation

\[
p^{\text{new}} = p^{k-1} + \alpha_p p'
\]

Where \( p' \) is pressure update, \( \alpha_p \) is a relaxation parameter between zero and one to ensure that small step sizes are taken between iterations. In order to update the velocity field, the algorithm employs different approach from the equation used to update the pressure field, taking only a fraction from both the previous field and the current field to calculate the new velocity field.
\[ u_{\text{new}} = \alpha_u u + (1 - \alpha_u) u^{k-1} \]
\[ v_{\text{new}} = \alpha_v v + (1 - \alpha_v) v^{k-1} \]

Equation 27

Where the \( u \) and \( v \) terms are found from Equation 24 and \( \alpha_u \) and \( \alpha_v \) are relaxation parameters as described above.

2.5 Check Convergence

If the correct velocity and pressure field were used from step 1, then the resulting pressure correction field calculated above would be zero everywhere. A second way to check for convergence is by calculating the percent difference between the velocity terms from the previous iteration to the current one with some minimum threshold \( \varepsilon \) to determine convergence.

Results

The Navier Stokes equation was solved using the SIMPLE algorithm in a structured grid and compared to the solutions by a commercial solver, FLUENT. Section 3.1 introduces the different parameters used for running the simulations. Section 3.2 explains the establishment of the fully developed flow between the plates. In section 3.3, the effect of fluid viscosity on the laminar flow will be shown. In section 3.4, the effect of the state value of velocity applied as a boundary condition will be discussed.

3.1 Dimensions and Boundary Conditions

Three different sets of parameters were implemented (Table 9) and the \( x \) and \( y \) velocity and pressure fields were compared in the structured and unstructured case. For all simulations, no slip boundary conditions were applied at the upper and lower walls, which should produce zero fluid velocity immediately at the walls. This is expected to create a parabolic velocity profile after the velocity profile is fully developed. In all simulations, velocity near the upper and lower walls should be zero. Outlet pressure boundary condition was kept the same in all cases (0.001Pa) and all simulations were run on a 5cm long by 5mm tall grid. The Reynolds number was calculated for each case, note that the Reynolds number was calculated using the ‘tube diameter,’ in this case the height of the rectangular mesh.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity, ( \mu )</td>
<td>1000</td>
<td>10</td>
<td>1000</td>
</tr>
<tr>
<td>Reynolds No.</td>
<td>1E-6</td>
<td>1E-4</td>
<td>5E-8</td>
</tr>
<tr>
<td>Inlet Velocity</td>
<td>0.2</td>
<td>0.2</td>
<td>0.01</td>
</tr>
</tbody>
</table>

3.2 The Fully Developed Profile

In flow through two parallel plates, when no slip boundary conditions are applied on the walls, the velocity profiles should be parabolic, where the maximum velocity is found near the center of the tube, and zero velocities are found near the upper and lower walls. When a flow is fully developed between two parallel plates, the \( x \) component of the velocity field \( U \) is determined by the inlet velocity \( U_{in} \), the entrance length \( d \) and the vertical position \( y \). These terms are connected by the following relationship,
\[ U(y) = 1.5 \cdot U_{in}(1 - \frac{4y^2}{d^2}) \] 

Equation 28

The vertical position \( y \) is equal to zero at the center between the upper and lower walls, and equals to \( \frac{1}{2} d \) at the upper wall and \( -\frac{1}{2} d \) at the lower wall, where \( d \) is the height of the geometry.

Figure 38 shows flow between two parallel plates at with constant inlet velocity of 0.2 m/s at the left boundary and a pressure of 0.001 Pa given at the right boundary. As expected, maximum velocity is observed at the center (except near the inlet), and zero velocity exists near the upper and lower walls due to the no slip boundary condition (Figure 38A). The \( x \) component of the velocity field forms nearly a flat profile near the inlet region, while away from the inlet it forms a parabolic profile. The region with parabolic \( x \) component of the velocity field is called the fully developed region, while the region near the inlet is characterized as the developing region.

Figure 38. MATLAB solution of flow between two parallel plates with a state boundary condition of a velocity inlet of 0.2 m/s and an outlet pressure of 0.001 Pa. (A) Shows the \( x \) component of the velocity field between the parallel plates, with maximum velocity near the center and zero velocity near the walls. (B) Shows a schematic of \( x \) component of the velocity field in the developing region near the inlet and in the developed region 6.5 cm into the tube. (C) Shows the \( y \) component of the velocity field between the parallel plates.

Figure 39 Flow between two parallel plates with inlet velocity = 0.2 m/s (top) and 0.01 m/s (bottom). Lowering the state value of the velocity inlet changes the magnitude but not the shape of the velocity profile.
In the developing region, the maximum x-velocity $U_{max}$ is 0.4 m/s, which is twice the inlet velocity. This ratio between maximum velocity and inlet velocity is predicted by Equation 28.

Figure 38C shows that y component of the velocity field is pointing towards the horizontal centerline in the developing region near the inlet. This observation supports the development of the parabolic profile. In the developing region, the velocity is establishing; in the fully developed region, a parabolic profile can be observed with $U_{max}$ equals to twice $U_{in}$ and $U_{min}$ (near the walls) equals to zero. In the fully developed region where the parabolic profiles are established, y velocities are zero everywhere.

In Figure 38C, the y component of the velocity field is shown; note that due to computational issues relating to limitations in MATLAB the solution is not fully converged as can be shown toward the outlet of the tube. In the developing region, the y component of the velocity field is pointing towards the horizontal centerline of the domain. In the fully developed region where the parabolic profiles are established, the y component of the velocity field is nearly zero everywhere.

3.3 Varying the Inlet Velocity

Looking at Equation 28, the maximum value of the x direction of the velocity field $U_{max}$ in the domain is proportional to the inlet velocity $U_{in}$. Using the code for unstructured grid, two different inlet velocities $U_{in}$ were applied at the left boundary and maximum values of the x velocity were compared against values predicted by the analytical Equation 28. Different inlet velocities ($U_{in}$) were used to examine the relationship between $U_{in}$ and $U_{max}$.

When $U_{in} = 0.2$ m/s is applied, $U_{max}$ is 0.317 m/s, and the ratio of $U_{max}/U_{in}$ is 1.585. When $U_{in} = 0.01$ m/s is applied, $U_{max}$ is 0.0156 m/s, and the ratio between $U_{max}/U_{in}$ is 1.56 (figure 3A, 3B). The ratio between $U_{max}$ and $U_{in}$ in both cases agrees with that predicted by Equation 28. $U_{in}$ and $U_{max}$ correlates linearly by a factor of 1.5.

3.4 Varying the Viscosity

The viscosity of the bulk fluid affects the viscous dissipation term in the Navier Stokes equation. In this section, fluid flow with two different viscosities (viscosity = 1000 Pa s and 10 Pa s) were compared to deduce its effect on parabolic flow development.
The respective velocity field and pressure drop for viscosity = 1000 Pa s and 10 Pa s (Figure 40A and B) are compared (Figure 40C). When the viscosity is 1000 Pa s, the maximum velocity in the x-direction is 0.4 m/s. When the viscosity is 10, maximum velocity in the x-direction is 0.7 m/s. When the fluid viscosity is smaller, higher flow velocities are reached.

Besides the difference in x velocity magnitudes, x velocity profiles along a vertical line is also qualitatively different (Figure 40C). The x velocity profiles are plotted at length = 0.025m from the inlet, where the velocities are fully developed and velocity in the y direction is zero. When the viscosity is 1000 Pa s, the x component of the velocity field forms a pronounced parabolic profile, where \( U_{\text{max}} \) equals two times \( U_{\text{in}} \); when viscosity is 10 Pa s, the x component of the velocity field has high gradient near the walls but is nearly flat in the center region.

In conclusion, high fluid viscosity affects the shape of the parabolic velocity profiles and also velocity magnitudes by attenuating the diffusion of velocity throughout the system.

Validation

The MATLAB code for solving Navier Stokes equation with the SIMPLE algorithm is validated with a commercial software, FLUENT. FLUENT is also based on principles of SIMPLE algorithm. The solutions of flow fields and pressure in FLUENT are obtained with convergence criteria of \( 10^{-6} \). Mesh dimensions for this validation is identical with that used in MATLAB. Boundary conditions are the ones described for Case 1 in Table 1.

4.1 Validation of MATLAB solution using FLUENT

The maximum x velocity in solutions generated by MATLAB and Fluent were 0.4 and 0.299 m/s, respectively as shown in Figure 41.
The y velocity obtained with both methods also showed good agreement (Figure 42 A and B). Near the inlet, y velocity shows large magnitudes as the parabolic velocity profiles are still developing. Y velocities away from the velocity inlet are zero everywhere as the fully developed velocity profile was established. Figure 40 shows the comparison of pressure drop across the domain.

**Discussion and conclusion.**

Numerical solutions of the Navier Stokes equation using the SIMPLE algorithm are very commonly used for the solution of fluid flow. In this chapter, the theoretical implementation of SIMPLE algorithm is demonstrated using MATLAB. The computation of fluid flow within parallel plates with no slip boundary results in a parabolic profile, as expected. The solution of the MATLAB program was validated with a commercial software, FLUENT. In the future, the species transport equation will be coupled to the Navier Stokes equation to solve for drug dispersion within a complex fluid flow field.
Appendix 2.

Simple sine wave function code used in the computational model. The code is interpreted by the “user defined functions” options in Fluent.

/***********************************************************************
UDF that DEFINE VELOCITY PROFILE (with loop)
***********************************************************************/

#include "udf.h"

DEFINE_PROFILE(velocityP_1, t, i)
{

    face_t f;

    real flow_time = RP_Get_Real("flow-time");

    begin_f_loop(f,t)
    {

        F_PROFILE(f,t,i)=0.01*sin(6.28318*flow_time);

    }

    end_f_loop(f,t)

}

Appendix B

The following case specifications contain detailed instructions to setup FLUENT simulations for those studies performed in this report.

Model A and B with constant flow
1. Import mesh into FLUENT [3dp]
2. GRID → SCALE: select ‘cm’
3. DEFINE → MODELS → SOLVER: select ‘steady’
4. DEFINE → MATERIALS → FLUENT DATABASE
a) Fluent database panel: 1) Select ‘fluid’
   2) Select “water”->select “copy”
   3) Close panel

6. **DEFINE → OPERATING CONDITIONS:** deactivate “gravity”

7. **DEFINE → BOUNDARY CONDITIONS:**
   a) Velocity inlet-> select “velocity inlet”
      (1) Momentum tab: set velocity magnitude to 0.005m/sec
   b) Pressure outlet-> select “pressure outlet”
      (1) Momentum tab: set gauge pressure to 0 Pascal
      (2) Species tab: set species mole fraction to 0

8. **SOLVE → INITIALIZE**
   a) Choose ‘Compute from inlet’

9. **SOLVE → ITERATE**
   a) Set Number of iteration to “500”
   b) Set Reporting interval to “1”
   c) Set UDF Profile Update Interval to “1”

-----------------------------------------------------------------------------------------------------------------------------

**Model A and B with pulsatile flow in 10 seconds**

1. **FINE → USER DEFINED → FUNCTIONS → INTERPRETED:**
   a) Read source file ‘fully developed sine wave’
   b) Interpret

2. **SOLVE.**
Appendix 3

The following case specifications contain detailed instructions to setup FLUENT simulations for pressure drop studies.

Case Specifications 1 (Without trabeculae)

Modeling the flow of water in the model with no obstructions
1. Import mesh into FLUENT [3ddp]
2. GRID --> SCALE: select ‘cm’
3. DEFINE --> MODELS --> SOLVER: select ‘steady’
4. DEFINE --> MATERIALS --> FLUENT DATABASE
a) Fluent database materials panel:
   1) Under ‘Fluent Fluid Materials’, select ‘water-liquid’
   2) Select ‘copy’
   3) Close panel
c) Fluent database panel: 1) Check ‘Name’ and ‘Material Type’ if they are ‘water-liquid’ and ‘fluid’ respectively
   2) Select ‘Change/Create’ and close the panel
6. DEFINE --> OPERATING CONDITIONS:
   a) Reference Pressure Location: X (m): -0.025
      Y (m): -0.005
      Z (m): 0.0015
7. DEFINE --> BOUNDARY CONDITIONS:
   a) Inlet --> select ‘velocity inlet’ --> Momentum tab: set the velocity magnitude to 0.01 m/s
   b) Outlet --> select ‘pressure outlet’ --> Momentum tab: set gauge pressure to 0 pascal
   c) Wall --> select ‘wall’
8. SOLVE --> MONITORS --> RESIDUAL
   a) Set the values as follows:
      (1) Iterations: 1000
      Absolute Criteria
      (1) Continuity 0.001
      (2) x-velocity 0.001
      (3) y-velocity 0.001
      (4) z-velocity 0.001
9. SOLVE --> INITIALIZE --> Click ‘Init’
10. SOLVE --> ITERATE --> Set number of iterations to 1000 --> Click ‘Iterate’

Case Specifications 2 (With trabeculae)

Modeling the flow of water in the model with obstructions
1. Import mesh into FLUENT [3ddp]
2. GRID --> SCALE: select ‘cm’
3. DEFINE --> MODELS --> SOLVER: select ‘steady’
4. DEFINE --> MATERIALS --> FLUENT DATABASE
a) Fluent database materials panel:
   1) Under ‘Fluent Fluid Materials’, select ‘water-liquid’
   2) Select ‘copy’
   3) Close panel
c) Fluent database panel:
1) Check 'Name' and 'Material Type' if they are 'water-liquid' and 'fluid' respectively
2) Select 'Change/Create' and close the panel

6. DEFINE -> OPERATING CONDITIONS:
   a) Reference Pressure Location: X (m): -0.005
      Y (m): 0.025
      Z (m): 0.0015

7. DEFINE -> BOUNDARY CONDITIONS:
   a) Inlet -> select 'velocity inlet' -> Momentum tab: set the velocity magnitude to 0.01
   b) Outlet -> select 'pressure outlet' -> Momentum tab: set gauge pressure to 0 pascal
   c) Wall -> select 'wall'

8. SOLVE --> MONITORS --> RESIDUAL
   a) Set the values as follows:
      1) Iterations: 1000
      2) Continuity 0.001
      3) x-velocity 0.001
      4) y-velocity 0.001
      5) z-velocity 0.001

9. SOLVE --> INITIALIZE --> Click 'Init'

10. SOLVE --> ITERATE --> Set number of iterations to 1000 --> Click 'Iterate'

-----------------------------------------------
Appendix 4

Appendix A

The following case specifications contain detailed instructions to setup FLUENT simulations for the studies performed in this report.

--------------------------------------------------------------------------------------------

Case Specifications (Middle point injection)

Modeling the flow of water in the model with obstructions

1. Import mesh into FLUENT [3ddp]
2. GRID → SCALE: select ‘cm’
3. DEFINE → User-defined→Function→Interpreted → ‘Source File Name: inlet_Cardiacvel_profile.c’ → click ‘Interpret’
4. DEFINE → MODELS → SOLVER: select ‘transient’
5. DEFINE → User-defined→Function→Interpreted → “Source File Name: inlet_Cardiacvel_profile.c” → click ”Interpret”
6. DEFINE → MODELS → Enable: Species Transport
7. DEFINE → MATERIALS → MIXTURE → FLUENT Database → select and copy water-liquid then acetone → back to Mixture panel ‘Material Type: Fluid’ → ‘FLUENT Fluid Materials: acetone’ → Change information of acetone. Name: Bupivacaine, Chemical Formula: Bupivacaine, Density: 998.2 (kg/m3), Viscosity: 0.001(kg/m-s), Molecular weight: 288.43 (kg/kgmol) → click ‘Change/create’
8. Double-click on ‘mixture-template’ → under ‘Mixture Species’ click ‘Edit’ → in ‘Selected Species’ box, arrange the species so that water-liquid is below Bupivacaine → click ‘OK’ → click ‘Change/create’

Note: make sure that Density is ‘volume-weight-mixing-law’, viscosity: 0.001 and diffusivity: 2.88e-10
9. DEFINE → OPERATING CONDITIONS:
   a) Reference Pressure Location: X (m): -0.005
      Y (m): 0.025
      Z (m): 0.0015

10. DEFINE → BOUNDARY CONDITIONS:
    a) Inlet → select ‘velocity inlet’ → Velocity inlet → edit → under Momentum tab, define velocity magnitude by clicking the drop down menu and select ‘udf velocity_in’ → under Species tab, select mass fraction of Bupivacaine to be 0.005
    b) Outlet → select ‘pressure outlet’ → Momentum tab: set gauge pressure to 0 pascal
    c) Wall → select ‘wall’
    d) Injection site → select ‘velocity inlet’ → Momentum tab: set the velocity to 4.71 cm/s.

Note: for the pressure outlet, make sure that Species mass fraction is zero
11. SOLVE → MONITORS → RESIDUAL
   a) Set the values as follows:
      1) Iterations: 1000

Absolute Criteria
1) Continuity          0.001
2) x-velocity          0.001
3) y-velocity          0.001
4) z-velocity          0.001

12. SOLVE → INITIALIZE → Click 'Init'

13. SOLVE → ITERATE → Set number of iterations to 1000 → Click 'Iterate'
Appendix 5. Parameter estimation with kinetic inversion techniques.

Abstract

Background: To model a complex system of biochemical reactions, all reaction rates need to be known. Sometimes it is not possible to determine all reaction rates of a biological system through experiments. Kinetic inversion problems involve estimating kinetic parameters of a reacting system with concentration measurements. The mathematical modeling of the reacting system defined by a set of differential equations lays the ground for the kinetic inversion problem, and the search for the right parameters that give the minimum error follows. In this report, the modeling of the reacting system with three species, A, B, and C, and three kinetic parameters, k1, k2 and k3 will be discussed. Then, assuming k1 and k2 are unknown, this report will illustrate the search for k1 and k2 with three different algorithms: steepest descent, sensitivity response surface, and global terrain tracking algorithm. The performance and robustness of the three algorithms will be covered.

Methods: The concentration trajectories of the reacting system defined by a set of differential equations are solved both analytically and numerically with known kinetic rates. The sensitivity information is calculated also both analytically and numerically. The analytical solution of the sensitivity information is calculated by the eigenvalue approach, where the differential equations of the sensitivity information are inhomogeneous. Then, we assume that k1 and k2 are unknown kinetic parameters, and that we have experimental concentration measurements of A, B and C over time. In this report, simulated 'perfect data' as well as 'perfect data with a random noise' will be used for the kinetic inversion problem to identify k1 and k2. Kinetic inversion problem is solved with the steepest descent algorithm, sensitivity response surface technique, and gradient method coupled with global terrain tracking method. The performance and robustness of the three algorithms are compared by testing a range of initial guesses. The topology of the residual error surface, the strengths and weaknesses of the algorithms are discussed.

Results: Concentration trajectories for A, B and C with known rates are computed. The analytical solutions of the concentration trajectories and the sensitivity trajectories are identified. The numerical solutions are compared to the analytical solutions. The eigenvalues of the sensitivity information are identical to the eigenvalues of the reacting species A, B and C. Steepest descent algorithm converges to the true solution when the initial guesses are within two times above or below the true solution. However, the sensitivity response surface technique converges to the true solution with a range of initial guesses up to 10^3 order above and 10^-5 below the true solution. Looking at the topology of the residual error surface, a valley is observed that leads to the true solution. Using the steepest descent algorithm coupled with global terrain tracking technique overcomes this limitation of poor range, tracks the valley and the robustness is improved. However, some knowledge of the residual error landscape is needed a priori which limits the general application of the global terrain technique.

Conclusion: Kinetic inversion problems can be solved by different numerical algorithms. Solving the kinetic inversion problem with sensitivity response surface technique is more robust than the steepest descent algorithm, since the sensitivity response surface technique admits a greater range of initial guesses. The limitation of the steepest descent algorithm can be overcome by coupling the algorithm with the global terrain tracking technique since we have some knowledge of the error landscape.
Introduction

Kinetic inversion enables the determination of kinetic parameters of a reacting system from experimental data. If the reacting system is represented by a set of differential equations, kinetic inversion technique begins from the computational modeling of the reacting system with a set of guessed parameter values. The reacting system with species A, B, and C are shown in Eqn 1, and the differential equations are shown in Eqn 2, Eqn 3 and Eqn 4:

\[
\begin{align*}
A & \overset{k_1}{\longrightarrow} B \overset{k_2}{\longrightarrow} C \\
\frac{\partial C_A}{\partial t} &= -k_1 \cdot C_A \\
\frac{\partial C_B}{\partial t} &= k_1 \cdot C_A - k_2 \cdot C_B + k_3 \cdot C_C \\
\frac{\partial C_C}{\partial t} &= k_2 \cdot C_B - k_3 \cdot C_C
\end{align*}
\]

Eqn 1

Eqn 2

Eqn 3

Eqn 4

If we do not know the kinetic rates \(k_1-k_3\), but we have concentration data of A, B, and C from experiments, we can determine the kinetic parameters through a technique called kinetic inversion. Kinetic inversion technique compares the simulated concentrations using different parameter sets with experimental measurements, and determine the error between simulated and experimental trajectories. The error values for using different combinations of kinetic parameters can be mapped into an error surface. The search of the right kinetic parameters happens on the error surface, and the right parameters are located at the minimum of the error surface. This process can be performed using rather basic algorithms such as the steepest descent method, or using more complex algorithms such as sensitivity response surface technique.

This report introduces three algorithms of solving the kinetic inversion problem: the steepest descent, the sensitivity response surface algorithms, and the global terrain valley-tracking method. First, the computational modeling of the reacting system described in Eqn 5, Eqn 6, Eqn 7, Eqn 8, will be discussed. Since constructing the sensitivity response surface requires the sensitivity information of the reacting system, the analytical and numerical computation of the sensitivity matrix will be covered. The third method, global terrain valley tracking method, takes advantage of the topology, or the landscape, of the residual error surface. The theory of interconnectedness states that all stationary points (local minimas, maximas, and saddle points) are connected through valleys and ridges.(91) If we take advantage of this interconnectedness and follow the valleys, we can identify all local minima in this kinetic inversion problem (Figure 43).

The topology of the error surface, the quality of the experimental data available and their combined effect on the convergence of the algorithms will be discussed. The performance of the two algorithms will be evaluated in terms of convergence rates and robustness. Lastly, the confidence intervals of the estimated parameters, \(k_1\) and \(k_2\), will be computed.
In this chapter, the introduction presents the reacting system of three species, and the concept of kinetic inversion. This report will cover six main topics, and the methodology and results section are each organized into these six main topics. The first topic of the methodology will discuss the solution of $C_A$, $C_B$, and $C_C$ with analytical and numerical techniques. The second topic will introduce the computation of sensitivity information of $A$, $B$, and $C$ in respect to each kinetic parameter. The third topic will introduce the residual error surface for a two parameter inversion, and the search for the minimum using the steepest descent algorithm. The fourth topic is dedicated to the sensitivity response surface method, which uses the sensitivity information to create a first-order approximated surface. The fifth topic introduces the global terrain methodology, which allows the search to follow the valleys and ridges of the surface. The sixth topic includes the computation of the confidence intervals for the estimated parameters. In the seventh topic, the kinetic inversion for a realistic reacting system, the transcription and translation of aquaporin channel, is performed. In the results section, the results for each topic are presented in the same order as in the methods section. The seventh topic in the results section contains a detailed investigation of the quality of the residual error surface. In the discussion section, the performance of the three algorithms, the topology of the residual surface, and the rational choice of inversion algorithm will be discussed. The conclusion will state the main findings of this report.

Methodology

**Topic 1: The analytical solution of $C_A$, $C_B$, $C_C$ by solving an eigenvalue problem.**

The eigenvalues of the matrix $A$ representing the system of equations is solved by Eqn 5, and the eigenvector corresponding to each eigenvalue is obtained by Eqn 6:

\[
\text{Det} (A - \lambda I) = 0 \quad \text{Eqn 5}
\]

\[
(A - \lambda I)x = 0 \quad \text{Eqn 6}
\]

According to Eqn 5, eigenvalues of matrix $A$ make matrix $A$ singular, hence determinant zero. The number of eigenvalues equals to the order of the system. If the matrix $A$ is already a singular matrix, there will be one eigenvalue that equals to zero. For Eqn 6, the only eigenvector will be the null vector if $(A - \lambda I)$ is a non-singular matrix. If $(A - \lambda I)$ is singular, which it should be since $\lambda$ are the correct eigenvalues, eigenvectors $x$ will be non-null (or non-trivial) vectors. The search of the eigenvectors for the stoichiometry matrix $A$ is related to the computation of the equilibrium of the reacting system. The eigenvalues and eigenvectors are used to assemble the analytical solutions for $C_A(t)$, $C_B(t)$, and $C_C(t)$.

**Topic 2: Sensitivity analysis of $C_A$, $C_B$, $C_C$ in respect to $k_1$, $k_2$, and $k_3$.**

Sensitivity information of $C_A$, $C_B$, $C_C$ in respect to kinetic parameters $k_1$, $k_2$, and $k_3$ can be expressed in a 3 by 3 matrix, $P$, as shown in Eqn 7, where the row elements of $P$ are sensitivity information of each species, and the column elements are sensitivity in respect to each parameter. As an example, $P_{A1}$ is the sensitivity of $C_A$ in respect to kinetic parameter $k_1$, and $P_{B1}$ is the sensitivity of $C_B$ in respect to kinetic parameter $k_1$. The system of equations of $C_A$, $C_B$, $C_C$ with 9 sensitivity equations results in a 12 by 12 matrix. This system can be solved analytically with an eigenvalue approach, or solved numerical by integration. Sensitivity information solved analytically and numerically will be compared in the results section.

\[
P = \begin{bmatrix}
P_{A1} & P_{A2} & P_{A3} \\
P_{B1} & P_{B2} & P_{B3} \\
P_{C1} & P_{C2} & P_{C3}
\end{bmatrix} \quad \text{Eqn 7}
\]

**Topic 3: Kinetic inversion with steepest descent algorithm**
Topic 3.1: The topology of residual surface with perfect and imperfect experimental data

For this problem $K_3 = 0.25$ is known while $k_1$ and $k_2$ need to be determined from data. For the kinetic inversion of 2 parameters $k_1$ and $k_2$, the unscaled residual surface is computed with the following formula in Eqn 8 for different realization of $k_1$ and $k_2$:

$$\sum (C_{\text{data}} - C_{\text{simulation}})^2$$  \hspace{1cm} \text{Eqn 8}

$C_{\text{simulation}}$ stands for simulated concentration trajectories for a set of $(k_1, k_2, k_3)$. Since we don’t know the true solution $(k_1^*, k_2^*)$, the simulated trajectories will perhaps be very different from the experimental data. For one set of $(k_1, k_2, k_3)$, Eqn 8 computes the error value (a scalar) by computing the sum of the squared errors between simulated and experimental concentrations over a period of time. Mapping the error values for different sets of $(k_1, k_2)$, we obtain a surface, where the height of the points on the surface represents the error magnitude, and the axes are the range of $k_1$ and $k_2$, respectively.

The quality of the data may affect the residual surface, and later will impact the convergence of the kinetic inversion problem. To illustrate this, the residual surface is plotted with perfect data (generated from simulation) and imperfect data (from simulation with an added random noise). The visibility of the minimum from visual inspection of the residual surface will be discussed.

Topic 3.2 Steepest descent algorithm

The steepest descent algorithm computes the descent direction on the error surface by using the first derivative information. The gradient is computed using the residual error values of 4 neighboring points along with the center point which is the current iterate $x^k$. The descent direction is the negative of the gradient. After the descent direction is obtained, the potential new iterate $(x^{pn})$ is computed as in

$$x^{pn} = x^k + \lambda d^{\text{descent}}$$ \hspace{1cm} \text{Eqn 9}

where $x^k$ is the current iterate, $\lambda$ is the step size, and $d^{\text{descent}}$ is the direction of descent. The steepest descent algorithm does an inexact line search along this direction of descent by comparing the model error value at new point $x^{pn}$ with the error value at original point $x^k$. If $x^{pn}$ has a higher error, the step size is contracted, and $x^{pn}$ is abandoned. If $x^{pn}$ has a lower error than $x^k$, then $x^{pn}$ is taken as the new current iterate.

Topic 4: kinetic inversion with sensitivity response surface algorithm

The simulation is performed at a guess $(k_1, k_2)$ and the sensitivity information of $(C_A, C_B, C_C)$ in respect to $(K_1, K_2)$ was derived as in Eqn 7. Using the sensitivity information at this point, the residual error surface for different $(k_1, k_2)$ sets was obtained as in Eqn 8. The minimum of this residual surface (the surface built with sensitivity information instead of direct simulation) can be solved iteratively by using the steepest descent algorithm. Once the solution is obtained, let’s call the minimum of this sensitivity error surface $(k_1^*, k_2^*)$. A new direct simulation with $(k_1^*, k_2^*)$ is conducted and new sensitivity is obtained with this set $(k_1^*, k_2^*)$. The sensitivity error surface is again solved iteratively for the minimum. This method requires less direct simulation than the steepest descent method shown previously, therefore may be computationally more efficient in large systems. A summary of the algorithm is reported in Table 10.

<table>
<thead>
<tr>
<th>Algorithm for sensitivity response surface technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algorithm for kinetic inversion using sensitivity information.</td>
</tr>
</tbody>
</table>
Step 1. Start with guess \((k_1 = 1.3, k_2 = 0.3)\);

Step 2. Solve for \(C_A, C_B, C_C\) and \(P_{A1}, P_{B1}, P_{C1}, P_{A2}, P_{B2}, P_{C2}, P_{A3}, P_{B3}, P_{C3}\) at \((k_1 = 1.3, k_2 = 0.3)\).

Step 3. Using sensitivity information, perturb both \(k_1\) and \(k_2\) by 0.001 to find the gradient.

Step 4. Taking the (-gradient) direction, update \(x_{\text{new}} = x_k + \lambda \ast (-\text{gradient})\).

Step 5. Find the lambda at this direction that makes the residual smaller than before (inexact line search).

Step 6. Staying on the sensitivity surface of \((k_1 = 1.3, k_2 = 0.3)\), keep on solving for \(x_{\text{new}}\) until minimum \((k_1 = k_1^*, k_2 = k_2^*)\) on this surface is found.

Step 7. At \((k_1^*, k_2^*)\), solve for \(C_A, C_B, C_C\) and \(P_{A1}, P_{B1}, P_{C1}, P_{A2}, P_{B2}, P_{C2}, P_{A3}, P_{B3}, P_{C3}\).

Repeat step 3-7. Until epsilon is met such that the error surface minimum is found.

### Topic 5. kinetic inversion with global terrain valley tracking method

The global terrain method can travel on the residual error surface along the valleys and ridges by using the second derivative, or the hessian, information. The eigenvalue decomposition of the hessian matrix reveals the eigen-directions of the terrain, which indicates the directions of the valleys and ridges.

#### Topic 5.1: Mathematical definitions of the terrain topology

For a residual error surface where the vertical axes is the residual error value, and horizontal axes are the parameters to be estimated for (Figure 44). A level set is composed of all the points on the residual error surface which have the same residual error value (L), hence the same ‘height’ on the surface. A level set is similar to the ‘iso-contours’ in a surface plot. Then, a valley is defined by a collection of points on a series of level sets, with maximum curvatures, as seen in Figure 45. These points with maximum curvatures can be identified by solving an constrained optimization problem on each level set with error L in Eqn 10.

**Mathematical Definition of a Ridge Point**

\[\text{Ridge point} = \{\max \text{ or } \min g^Tg \text{ s.t. } F^TF = L, \text{for all } L \in L\}\]

Where \(g = 2J^TF, J\) is the Jacobian of F, L is a given value of the error, so \(F^TF = L\) means all points on the level curve has error magnitude of L. L is a collection of curves, each with a different error magnitude.

The constraint for this optimization problem limits the search on a specific level set with error value L, as in Eqn 11:

\[F^TF = L\]

The necessary condition for this optimization problem is:

\[Hg - \lambda g = 0\]

where H is the 2nd derivative of the function F, and \(\lambda\) is the Lagrangian multiplier associated to Eqn 10, the level constraint. However, in this kinetic inversion problem, we do not have an analytical objective function. Therefore, the first and second derivatives are not found by the analytical differentiation of the objective function. These derivatives are found by using the scalar values of the surface. The hessian information for the residual scalar surface, is computed using the residual error values by doing a small perturbation in each unknown kinetic parameter, and the grid of perturbation is shown in Figure 46. The computation of the gradient and the hessian on this scalar residual error surface will be described in the next section.

#### Topic 5.2 Computation of the hessian information from a scalar residual surface

The topology of a smooth and differentiable surface is shown in Figure 44, and the associated level sets and the local minima are shown in Figure 45. The direction of the valley, starting from one of the local
minimum, is shown in Figure 45 with red arrows. This direction can be derived from the second derivative, or the hessian, of the scalar surface at the current iterate. The computation of residual error values for 8 neighboring points is necessary for the hessian information, see Figure 46. The neighboring points are chosen by doing a small perturbation of each parameter in both directions. The magnitude of the perturbation is referred to as the perturbation distance.

For the residual error surface of two unknown parameters, the computation of the gradient at point 5 in Figure 46 only requires 4 neighboring points, and an example of the gradient in k1 direction is shown in Eqn 13. The computation of the hessian, requires 8 neighboring points. The second derivative of R in respect to k1 is shown in Eqn 14:

\[
\frac{\partial R}{\partial^2 k1} = \frac{(R2 - R5)}{(1 \cdot \text{perturbation distance})} - \frac{(R5 - R8)}{(1 \cdot \text{perturbation distance})} \cdot \frac{1}{1 \cdot \text{perturbation distance}}
\]

Eqn 14

\[
\text{Gradient in } k1 \text{ direction} = \frac{R2 - R8}{2 \cdot \text{perturbation distance}}
\]

Eqn 13

![Figure 44] A smooth surface will multiple local minima. Global terrain methods take advantage of the interconnected valleys of the surface and find all local minima. Following the valleys (black arrows) requires the hessian information of the residual error surface.

![Figure 45] A set of level curves and three local minima. The eigenvalues at the uppermost local minimum (red dot) indicate the movement direction of the algorithm. The sequence of red arrows signal the direction of the valley connecting to the next local minimum.

![Figure 46] Computation of the gradient and hessian of the residual error surface using a nine-point stencil. The residual error values for 8 points around the current iterate, point 5, are computed for a small perturbation of k1 and k2, independently. The gradient at point 5 is computed using point 2,4,6, and 8; the hessian is computed using all the surrounding 8 points.

**Topic 5.3: eigen-value decomposition of the hessian**

Once the hessian is obtained at an iterate, the terrain direction information is hidden in the eigenvalues and eigenvectors of the hessian matrix. The eigenvalue decomposition of a matrix is shown previously in Eqn 5 and Eqn 6. Since we are searching for two parameters, there will be two eigenvalues and two eigenvectors. To follow a valley with either uphill or downhill movements, the eigenvalue with the smallest absolute value should be chosen. The eigenvector corresponding to this eigenvalue is the direction
that the algorithm takes. If the bottom of the valley is going slightly uphill, the sign of this eigenvalue will be positive. If the valley is going slightly downhill, the sign of this eigenvalue will be negative. In either case, the eigenvalue with the smallest absolute value indicates the direction of a valley. In the results section, examples will be given illustrating this concept with different sample points on the residual surface.

**Topic 6: Confidence intervals for k1 and k2**

The kinetic inversion algorithms generate point estimates of the unknown parameters, without revealing how much 'confidence' we have about the point estimates. The 'confidence' that we have about the point estimates is related to the error between the model and the data, and the noise within the data itself. However, this noise is not reflected by the point estimates of the kinetic parameters. Even when the experimental data quality is bad and the noise is high, the algorithm will still report an unique minimum without taking the noise in the data and the error of the present model into consideration. Therefore, it is better to say that we have 90% confidence that k1 will fall within a certain 'range' around the point estimates. This range, termed the confidence interval, can be computed from the variance and covariance in the estimated parameters. If only the variances for the individual parameters are considered, the range is called the individual confidence region. If the estimated parameters are co-related, and both the variances and covariances are considered, the confidence range will be referred to as the joint confidence region.

**Topic 6.1: Individual confidence region**

Individual confidence region considers only the variances of the parameters, and it does not consider the covariances between parameters. This concept will be illustrated in the following section. The matrix of variances and covariances are computed according to Eqn 15

\[ V_{\text{parameter}} = s^2 (J^T J)^{-1} \]  

where \( s^2 \) is the model error divided by the degree of freedom of the system as in Eqn 16, \( J \) is the Jacobian of the states (concentrations) in respect to each parameter \( k_1 \) \( k_2 \), which is equivalent to the sensitivity information in this case. The Jacobian (sensitivity of \( C_A, C_B, C_C \) in respect to \( k_1, k_2 \)) is shown in Eqn 17:

\[ s^2 = \frac{\sigma^2}{n-p} = \frac{0.01319}{94-3-2} = 0.0000471 \]  

\[ \text{jacobian} = \begin{bmatrix} \frac{\partial C_A}{\partial k_1} & \frac{\partial C_A}{\partial k_2} \\ \frac{\partial C_B}{\partial k_1} & \frac{\partial C_B}{\partial k_2} \\ \frac{\partial C_C}{\partial k_1} & \frac{\partial C_C}{\partial k_2} \end{bmatrix} \]  

For this kinetic inversion problem, \( J^T J \) is shown in Eqn 18, and the variance is a 2 by 2 matrix shown in Eqn 19:

\[ J^T J = \begin{bmatrix} 27.38 & 14.123 \\ 14.123 & 537.47 \end{bmatrix} \]  

\[ V_{\text{parameter}} = \begin{bmatrix} 0.174 \cdot 10^{-5} & -0.00458 \cdot 10^{-5} \\ -0.00458 \cdot 10^{-5} & 0.00888 \cdot 10^{-5} \end{bmatrix} \]  

For the computation of individual confidence region, only the diagonal elements (variances) of matrix \( V \) are used. In the next section, the covariances (off-diagonal elements) along with the variances (diagonal elements) are both used for the computation of joint confidence region.

**Topic 6.2: Joint confidence region**

In contrast to individual confidence region, the joint confidence region considers the variances as well as the covariances of the parameters. This means that the correlations of the parameters are taken into consideration. In this case, reporting the joint confidence region for \( k_1 \) and \( k_2 \) will be more appropriate, since \( k_1 \) and \( k_2 \) are co-related. The formula for deriving the joint confidence region is shown in Eqn 20:
Eqn 20

\[(k_1 - \bar{k}_1)^T V_{\text{parameter}}^{-1} (k_1 - \bar{k}_1) \leq p \cdot F\]

where \(\bar{k}_1\) is the point estimate of \(k_1\), \(V\) is the variance matrix, \(p\) is the number of parameters estimated, and \(F\) is the F-score from F-distribution. Eqn 20 is the equation for an ellipse, where the directions of the main axes are the eigenvectors, and the length of these axes are the corresponding eigenvalues.

**Topic 7. Case study: Parameter estimation for aquaporin transcription and translation rates**

To solve a realistic parameter estimation problem, a reaction network describing all steps of the transcription and translation of aquaporin channels is formulated based on published literature in cellular biology and protein synthesis. The aquaporin channel is abundantly expressed in many cells inside the body such as kidney, lungs, and the brain. This channel specializes in the transport of water and the maintenance of osmotic pressure across the cell. We want to estimate unknown transcription and translation rates of aquaporin production network in a cell.

**Topic 7.1. Governing equations of aquaporin transcription and translation network**

The rate of transcription and translation of a target protein in a cell involves the concentrations of participating reactants and enzymes. For example, the rate of transcription is dependent upon the concentrations of transcription factors and RNA polymerases in the nucleus compartment. Thus, transcription and translation rates can be formulated in terms of reactant concentrations, \(C_i\) and reaction rates, \(k_i\). In the following chemical formulas, ‘\(A \cdot B\)’ indicates A bound to B, or AB complex. Double arrows are reversible reactions, and single arrows are irreversible reactions. The chemical formulas are as follows:

\[
\begin{align*}
\text{gene} + TF1 & \xleftrightarrow[k_1/ k_2]{} TF1 \cdot \text{gene} \\
TF1 \cdot \text{gene} + TF2 & \xleftrightarrow[k_3/ k_4]{} TF2 \cdot TF1 \cdot \text{gene} \\
RNAP + TF2 \cdot TF1 \cdot \text{gene} & \xleftrightarrow[k_5/ k_6]{} RNAP \cdot TF2 \cdot TF1 \cdot \text{gene} \\
RNAP \cdot TF2 \cdot TF1 \cdot \text{gene} & \xleftrightarrow[k_7]{} TF2 \cdot TF1 \cdot \text{gene} + RNAP + RNA \text{ nuc} \\
RNANuc + Exportin & \xleftrightarrow[k_8/ k_9]{} RNANuc \cdot Exportin \\
RNANuc \cdot Exportin & \xrightarrow[k_{10}]{} RNAcytoplasm + Exportin \\
RNAcytoplasm + Ribosome & \xleftrightarrow[k_{11}/ k_{12}]{} RNA \cdot \text{ribosome} \\
RNA \cdot \text{ribosome} & \xrightarrow[k_{13}]{} RNAcytoplasm + Ribosome + UnfoldedProtein \\
UnfoldedProtein & \xrightarrow[k_{14}]{} \text{AquaporinChannel}
\end{align*}
\]

These concentrations of each species in these chemical formulations can be expressed as a set of differential equations as functions of time:

\[
\begin{align*}
\frac{d[TF1]}{dt} &= k_2[TF1 \cdot \text{gene}] - k_1[\text{gene}][TF1] \\
\frac{d[TF2]}{dt} &= k_4[TF2 \cdot TF1 \cdot \text{gene}] - k_3[TF2][TF1 \cdot \text{gene}] \\
\frac{d[\text{gene}]}{dt} &= k_2[TF1 \cdot \text{gene}] - k_1[\text{gene}][TF1]
\end{align*}
\]
\[ \frac{\partial [TF1 \cdot gene]}{\partial t} = k1[\text{gene}][TF1] + k4[TF2 \cdot TF1 \cdot gene] - k2[TF1 \cdot gene] - k3[TF2][TF1 \cdot gene] \]

Eqn 33

\[ \frac{\partial [TF2 \cdot TF1 \cdot gene]}{\partial t} = k3[TF1 \cdot gene][TF2] + k6[RNAP \cdot TF2 \cdot TF1 \cdot gene] + k7[RNAP \cdot TF2 \cdot TF1 \cdot gene] - k4[TF2 \cdot TF1 \cdot gene] - k5[RNAP][TF2 \cdot TF1 \cdot gene] \]

Eqn 34

\[ \frac{\partial [RNAP \cdot TF2 \cdot TF1 \cdot gene]}{\partial t} = k5[RNAP][TF2 \cdot TF1 \cdot gene] - k6[RNAP \cdot TF2 \cdot TF1 \cdot gene] - k7[RNAP \cdot TF2 \cdot TF1 \cdot gene] \]

Eqn 35

\[ \frac{\partial [RNAP]}{\partial t} = k6[RNAP \cdot TF2 \cdot TF1 \cdot gene] + k7[RNAP \cdot TF2 \cdot TF1 \cdot gene] - k5[RNAP][TF2 \cdot TF1 \cdot gene] \]

Eqn 36

\[ \frac{\partial [RNA\text{nuc}]}{\partial t} = k7[RNAP \cdot TF2 \cdot TF1 \cdot gene] - k8[\text{Exp}] + k9[RNA\text{nuc} \cdot Exportin] \]

Eqn 37

\[ \frac{\partial [\text{Exportin}]}{\partial t} = -k8[RNA\text{nuc}][\text{Exportin}] + k9[RNA\text{nuc} \cdot Exportin] + k10[RNA\text{nuc} \cdot Exportin] \]

Eqn 38

\[ \frac{\partial [RNA\text{nuc} \cdot Exportin]}{\partial t} = k8[\text{Exp}][RNA\text{nuc}] - k9[RNA\text{nuc} \cdot Exportin] - k10[RNA\text{nuc} \cdot Exportin] \]

Eqn 39

\[ \frac{\partial [RNAc\text{yto}]}{\partial t} = v_{\text{nuc}}[\text{nuc}] - k10[RNA\text{nuc} \cdot Exportin] - k11[RNAc\text{yto}][\text{Ribosome}] + k12[RNAc\text{yto} \cdot \text{Ribosome}] + k13[RNAc\text{yto} \cdot \text{Ribosome}] + k17[RNAc\text{yto}] \]

Eqn 40

\[ \frac{\partial [\text{Rib}]}{\partial t} = k12[RNAc\text{yto} \cdot \text{Ribosome}] - k11[RNAc\text{yto}][\text{Ribosome}] + k13[RNAc\text{yto} \cdot \text{Ribosome}] \]

Eqn 41

\[ \frac{\partial [RNAc\text{yto} \cdot \text{Ribosome}]}{\partial t} = k11[RNAc\text{yto}][\text{Ribosome}] - k12[RNAc\text{yto} \cdot \text{Ribosome}] - k13[RNAc\text{yto} \cdot \text{Ribosome}] \]

Eqn 42

\[ \frac{\partial [Unfolded\text{Protein}]}{\partial t} = k13[RNAc\text{yto} \cdot \text{Ribosome}] - k14[Unfolded\text{Protein}] - k15[Unfolded\text{Protein}] \]

Eqn 43

\[ \frac{\partial [Aquaporin]}{\partial t} = k14[Unfolded\text{Protein}] - k16[Aquaporin] \]

Eqn 44

The kinetic rates k1-k17 and the role of these rates are listed in Table 11:

<table>
<thead>
<tr>
<th>rates</th>
<th>significance</th>
<th>value</th>
</tr>
</thead>
<tbody>
<tr>
<td>k1</td>
<td>Binding rate of TF1 and gene</td>
<td></td>
</tr>
<tr>
<td>k2</td>
<td>Dissociation rate of TF1 from gene</td>
<td></td>
</tr>
<tr>
<td>k3</td>
<td>Binding of TF2 to TF1 - gene</td>
<td></td>
</tr>
<tr>
<td>k4</td>
<td>Dissociation of TF2 from TF1 - gene</td>
<td></td>
</tr>
<tr>
<td>k5</td>
<td>Binding of TF2 - TF1 - gene to RNA polymerase</td>
<td></td>
</tr>
<tr>
<td>k6</td>
<td>Dissociation of RNA polymerase from TF2 - TF1 - gene</td>
<td></td>
</tr>
<tr>
<td>k7</td>
<td>transcription rate</td>
<td>unknown</td>
</tr>
<tr>
<td>k8</td>
<td>binding rate of RNA in the nucleus</td>
<td></td>
</tr>
<tr>
<td>k9</td>
<td>Dissociation of RNA in the nucleus</td>
<td></td>
</tr>
<tr>
<td>k10</td>
<td>Release rate of RNAcyto from exportin in the cytoplasm</td>
<td></td>
</tr>
<tr>
<td>k11</td>
<td>Binding rate of RNAcyto to ribosome</td>
<td></td>
</tr>
<tr>
<td>k12</td>
<td>Dissociation rate of RNAcyto from ribosome</td>
<td></td>
</tr>
<tr>
<td>k13</td>
<td>translation rate</td>
<td>unknown</td>
</tr>
<tr>
<td>k14</td>
<td>immature protein folding rate</td>
<td></td>
</tr>
</tbody>
</table>
The reaction model is integrated for the first 2 hours, while the model reaches the steady state. The initial concentrations given are: 5 for TF1, 5 for TF2, 0.0001 for gene, 5 for RNA polymerase, 5 for exportin, 5 for ribosomes, and zero for the rest of the species. The solver used in MATLAB for integration of the differential equations is ODE15s, which is a semi-implicit solver.

This transcription and translation network has 15 species, whose concentrations are characterized by 15 differential equations with nonlinear terms. We will try to estimate two unknown parameters, $k_7$, transcription rate, and $k_{13}$, translation rate, from this network. Synthesized data for some species will be used to compare model error. The purpose of this exercise is not only to discover unknown kinetic rates, but also to assess the quality of the residual surface as a function of data availability. Out of the biochemical 15 species, how many species do we need to measure experimentally to determine two kinetic rates with reasonable accuracy?

**Topic 7.2. Experimental design based on parameter estimation**

In reality, the concentration data of intracellular species such as transcription factors and ribosomes are difficult to obtain. Often, the ability to estimate reaction rates are hindered by the lack of data. Usually, the availability of concentration data sets is limited to a mere few species. Let’s assume that we are designing an experiment to measure species concentration trajectories in order to estimate unknown kinetic parameters. There is no need to obtain concentrations trajectories of all 15 species in order to estimate two unknown kinetic rates. The question becomes, how many sets of concentration data are absolutely necessary? At first, we will use the concentration data of only two species, mRNA-cytoplasm, and aquaporin on the membrane, to estimate two parameters. The number of available data will be increased to include three, six and nine species. The quality of the respective residual error surfaces are analyzed in terms of data availability. This is crucial in designing an experiment to measure the least amount of species necessary for the determination of unknown rates.

**Topic 7.3 Kinetic inversion during dynamic transitions**

To investigate whether the residual error surface is better conditioned when the reacting system is undergoing dynamic transition due to an external input, a dynamic transition phase is introduced into the system by a temporary decrease in the RNA transport from the nucleus to the cytoplasm. When the time is between 15000 and 18000 seconds, the binding rate of RNA in the nucleus to exportin is decreased by 70%. This would cause an expected decrease in the transport rate of RNA out of the nucleus. This transient decrease is executed by the following code in MATLAB shown in Eqn 45.

```matlab
if 15000 < t && t < 18000
    k8 = 0.3*k8;
end
```

For this reacting system undergoing dynamic transition, the sum of squared error is computed over 6 hours and the residual error surface was constructed. The residual error surfaces built from using 6 data sets and 9 data sets are compared.
Results

**Topic 1: The analytical solution of CA, CB, CC by solving an eigenvalue problem**

**Topic 1.1: Trajectories of CA, CB, CC**

The analytical solutions of $C_A$, $C_B$, $C_C$ are assembled after obtaining the eigenvalues and eigenvectors:

\[ CA(t) = 3e^{-t} \]  
\[ CB(t) = (-9)e^{-t} + (26/3)e^{-0.75t} + 0.75 \]  
\[ CC(t) = 6e^{-t} - (26/3)e^{-0.75t} + 8/3 \]

The trajectories of $C_A$, $C_B$, $C_C$ are shown in Figure 47.

![Figure 47. Trajectories of species A, B and C over 30 seconds of time. A reacts irreversibly to B, and A is depleted over time. B and C reach equilibrium after 10 seconds.](image)

**Topic 1.2: Comparison of analytical to the numerical solution by integration of ODE.**

The trajectories produced by analytical and numerical solutions of A, B and C are identical.

**Topic 2: Sensitivity analysis of CA, CB, CC in respect to $k_1$, $k_2$ and $k_3$**

**Topic 2.1: Analytical solution of sensitivity trajectories by eigenvalue problem and eigenvalue multiplicity**

The three species and nine sensitivity equations are set up to form a 12 by 12 matrix. The 12 eigenvalues are shown in Figure 48.
There are 3 unique eigenvalues, -0.75, 0, and -1.0, each with a multiplicity of 4. The three unique eigenvalues are the eigenvalues for the stoichiometry matrix of the reacting system, and the multiplicity arises from the addition of the sensitivity equations. The new eigenvalues for the 9 sensitivity equations are identical to the original 3 eigenvalues for the three species \( C_A, C_B, \) and \( C_C \). The sensitivity information of the reacting species and the species themselves have the same eigenvalues. The method to solve eigenvectors for eigenvalues with multiplicity is included in the appendix file of this report. For example, with a multiplicity of 2, the second eigenvector \( x_2 \) is obtained by solving Eqn 47.

\[
(A\lambda I)^2 x_2 = 0
\]

Eqn 47

The eigenvalues and eigenvectors of the sensitivity information can be used to assemble the analytical solution of the sensitivity information. The analytical expression of \( P_{A1} \), the sensitivity of \( C_A \) in respect to \( k_1 \), is shown in Eqn 48:

\[
P_{A1} = -3te^{-t}
\]

Eqn 48

The shape of the analytically solved sensitivity trajectory is identical to the shape of the numerically solved sensitivity information of \( P_{A1} \).

**Topic 2.2: Comparison of analytical to numerical solution**

The sensitivity of \( C_A, C_B, C_C \) in respect to \( k_1, k_2 \) and \( k_3 \) are plotted over 30 seconds of time (Figure 49).
Figure 49. Sensitivity of species A, B, and C in respect to k1, k2, and k3 over time. The sensitivity values reach a constant after some time, when equilibrium is established. When the sensitivity curve lies at zero, it means that the concentration of that species is not influenced by this parameter. When the curve lies above zero, it means the concentration is increased by this parameter; conversely, when the curve lies below zero, it means that the concentration decreases in response to this parameter.

**Topic 2.3: The effect of perturbation of k1 and k2 on concentrations of A, B, and C**

Since we have obtained sensitivity matrix P, we can now perturb k1 and k2 and observe the effect of the perturbation on the concentration trajectories. Namely, we are changing the kinetic rates k1 and k2 slightly from the nominal values and see how the simulated concentrations are different from before (Figure 50).

For large perturbations of kinetic parameters, the sensitivity information may produce inaccurate concentrations. In the following figure, trajectories from sensitivity information are compared to those from direct simulation. First, a small perturbation is done (Figure 51): the new trajectories of A, B, and C predicted using sensitivity information are indistinguishable from those of resimulation. When a 100% perturbation of ka is done (ka = 1.0 → ka = 2.0), it can be observed in Figure 52 that the new trajectories using sensitivity information are far apart from those by resimulation.
Figure 50. Perturbation of k2 and k3 while k1 is fixed. Original parameter set is (1.0, 0.5, 0.25) and new parameter set is (1.0, 0.55, 0.2). The new trajectories (line with star) are derived using the sensitivity information. It can be observed that after the perturbation, the equilibrium concentrations of species B and C are shifted.

Figure 51. Comparison of original trajectories, resimulation trajectories and trajectories generated by sensitivity analysis for a small perturbation of k1. When the new parameter set (1.1, 0.5, 0.25) doesn't defer greatly from the old set (1.0, 0.5, 0.25), the trajectories from sensitivity analysis and resimulation are no different from each other. The sensitivity predicted concentration trajectories are accurate.
Figure 52. Comparison of original trajectories, resimulation trajectories and trajectories generated by sensitivity analysis for a large perturbation of k1. When the new parameter set (2.0, 0.5, 0.25) defers greatly from the old set (1.0, 0.5, 0.25), the trajectories from sensitivity analysis are very different from those of resimulation, indicating that the prediction generated by sensitivity analysis are not accurate when the perturbation of parameter is too great.

**Topic 3: Kinetic inversion with steepest descent algorithm**

#### 3.1.1 Error surface using Perfect Data
Perfect data (from simulation) is used as experimental data in this case. The error between experimental data and direct simulation with different realizations of (ka, kb) is computed. The sum of squared error is plotted in Figure 53, where the color intensity shows the error magnitude for different realizations of (ka, kb). The log of sum of squared error is plotted in Figure 54, where a minimum can be clearly observed. The minimum gives the solution (ka, kb).

#### 3.1.2 Error Surface using Imperfect Data
For this problem, a random number generator was used to introduce an error term between [-0.5, 0.5] into the perfect data. The effect of the magnitude of the random error was studied by multiplying the random error with a constant. Pseudo experimental data with error of magnitude 1x, 10x, and 100x, was generated. The comparison of the residual surface of perfect data and imperfect data is shown in Figure 55(1x error), Figure 56(10x error), Figure 57 (100x error), and Figure 58(different error magnitudes for each species).
Figure 53 Topology of residual error surface for $k_1 = 0.1-2.0$ and $k_2 = 0.1-1.0$ using perfect data set. Color intensity shows the magnitude of squared error. This minimization problem is solved by the steepest descent algorithm.

Figure 54. Log of sum of squared error for $k_a = 0.1-2.0$ and $k_b = 0.1-1.0$ using perfect data set. A sharp minimum is observed. The minimum gives the solution of $(k_1, k_2)$.

+ 

The surface of logarithm('sum of squared error') using perfect data shows a sharp minimum which marks the solution of $k_1$ and $k_2$. When a random error is introduced into the data, the quality of the residual surface is reduced since the minimum of the surface is no longer sharp and clear. In this example, the errors introduced into $C_A$, $C_B$ and $C_C$ are independently generated; the errors of each state are not co-related. The equation for the random error generation is shown in Eqn 49 and Eqn 50.

$$C_{\text{imperfect data}} = C_{\text{perfect data}} + \text{noise};$$  

Eqn 49

$$\text{noise} = (\text{random number between -0.5 and 0.5 following normal distribution})/100;$$  

Eqn 50

When the error is magnified, the minimum of the residual surface becomes very round, although there is still an unique minimum.
Figure 55. Log of error plot for perfect data (top) and data with experimental error (bottom) (Error = x).

Figure 56. Log of error plot for perfect data (top) and data with experimental error (bottom) (Error = 10x). The magnitudes of the error introduced are 10 times greater than that in Figure 1.

Figure 57. Error plot for data with experimental error (Error = 100x). The error surface using perfect data (top subplot) has a sharp and clear minimum point. When an error of 100x magnitude (compared to Figure 3) is introduced into Ca, Cb and Cc, the minimum of the error surface becomes hard to identify. This means that multiple sets of (k1, k2) may be identified as possible solutions.

Figure 58. Error plot for data with experimental error of uneven magnitudes for each species (Error for Ca and Cc = x, error for Cb = 100x). The error surface does not have a sharp minimum.

**Topic 3.2: Performance of steepest descent algorithm**

The error surface is a quadratic scalar field. The minimum can be found by solving the minimization problem with the steepest descent algorithm. The algorithm starts with a guess (k1 = 1.1, k2 = 0.45) that is relatively close to the true solution (1.0, 0.5), computes the steepest descent direction at this point, and finds the step size such that the error is reduced compared to the original point. As an example, iterations of x^k and the gradient at x^k for each iteration is shown in Table 1 with the starting point (1.1, 0.45). The algorithm converged in 5 iterations (A very relaxed epsilon is used to demonstrate the converging series of x^k in a short table).

**Table 12.**

<table>
<thead>
<tr>
<th>Iteration</th>
<th>Gradient at x^k</th>
<th>New x^k</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.003421943178108</td>
<td>1.095722571027366</td>
</tr>
<tr>
<td>Epsilon is met</td>
<td>Minimum of error surface ((ka = 1.066, kb = 0.507)).</td>
<td></td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------------------------------------------------------</td>
<td></td>
</tr>
</tbody>
</table>

**Topic 4: kinetic inversion with sensitivity response surface algorithm**

**Topic 4.1: Performance of sensitivity response surface algorithm**

The first 10 sensitivity surfaces and their respective minima are shown in Table 13. The kinetic inversion algorithm starting at (1.1, 0.45) using sensitivity information converged in 120 steps, where 120 sensitivity matrices were computed. The error surface estimated by the sensitivity information of the first point (1.1, 0.45) is shown in Figure 12. As a comparison, the residual error surface generated by direct simulation is shown in Fig 13. The solution found by solving the kinetic inversion using sensitivity is (\(ka = 1.06148332617580, kb = 0.499435526625092\)).
Table 13: First 10 iterations of kinetic inversion starting at \((ka = 1.1, kb = 0.45)\) showing convergence on 10 sensitivity surfaces (each row is one re-evaluation of the function and sensitivity matrix)

<table>
<thead>
<tr>
<th>Real function evaluation times</th>
<th>Minimum of this sensitivity surface</th>
<th>steps needed on this sensitivity surface =</th>
<th>Minimum of this sensitivity surface</th>
<th>steps needed on this sensitivity surface =</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.048334787057541</td>
<td>53</td>
<td>1.044879553351052</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0.495298891536744</td>
<td></td>
<td>0.498270229720091</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.039912993273837</td>
<td></td>
<td>0.032298139749169</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.044203886181321</td>
<td>2</td>
<td>1.043538805078910</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4968402020772410</td>
<td></td>
<td>0.49843153481185810</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.031352952810645</td>
<td></td>
<td>0.030458418508468</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.042882201181351</td>
<td>2</td>
<td>1.042234120969992</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.498439581483470</td>
<td></td>
<td>0.498468617793558</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.029593839711248</td>
<td></td>
<td>0.028755679447028</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.041594555683955</td>
<td>2</td>
<td>1.041594555683955</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.498492447220826</td>
<td></td>
<td>0.498492447220826</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.029593839711248</td>
<td></td>
<td>0.029593839711248</td>
<td></td>
</tr>
<tr>
<td>real function evaluation times</td>
<td>Minimum of this sensitivity surface</td>
<td>Iterations...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------------------</td>
<td>---------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.040963463494334</td>
<td>Solution of ( k_a ), ( k_b )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>steps needed on this sensitivity surface = 2</td>
<td>0.498513065500572</td>
<td>1.006148332617580</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>error at minimum</td>
<td>0.499435526625092</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.027150745452136</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>real function evaluation times</td>
<td>1.040340782834150</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.498531673790994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>steps needed on this sensitivity surface = 2</td>
<td>error at minimum</td>
<td>0.026381509175961</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.026381509175961</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>real function evaluation times</td>
<td>1.039726440422303</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.498548995454255</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>steps needed on this sensitivity surface = 2</td>
<td>error at minimum</td>
<td>0.025633271712074</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.025633271712074</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>real function evaluation times</td>
<td>1.006148332617580</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0.499435526625092</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>steps needed on this sensitivity surface = 2</td>
<td><strong>Solution of</strong> ( k_a ), ( k_b )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Solution of</strong> ( k_a ), ( k_b )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.006148332617580</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.499435526625092</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The robustness of the sensitivity response surface technique is tested by using initial guesses that are several orders of magnitude away from the true solution \((k_1 = 1.0, k_2 = 0.5)\).

Table 14 shows that the algorithm converge to the right solution with starting points as small as \(10^{-5}\) times smaller than true solution, and \(10^3\) larger than the true solution. The robustness of the algorithm improved drastically compared to the steepest descent algorithm, and the range of the initial guesses this algorithm admits is as wide as \(10^8\) (from \(10^{-5}\) below to \(10^3\) above true solution). The sensitivity response surface technique allows the inversion of kinetic parameters from data without prior knowledge of the values of the unknown parameters.

Table 14. Performance of the kinetic inversion algorithm with sensitivity response surface technique from different initial guesses

<table>
<thead>
<tr>
<th>Starting point ((k_1, k_2))</th>
<th># of iterations required</th>
<th>Final solution of inversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>((100, 50))</td>
<td>2000</td>
<td>0.998214427947730 0.499657307516817</td>
</tr>
<tr>
<td>((2000, 2000))</td>
<td>50,000</td>
<td>0.998526221137590 0.499648678941057</td>
</tr>
<tr>
<td>((0.0001, 18))</td>
<td>100</td>
<td>0.998217990650409</td>
</tr>
<tr>
<td>Starting Point</td>
<td>Computational Time</td>
<td>Sensitivity Value</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>(0.0001, 0.0001)</td>
<td>10,000</td>
<td>0.998216374792185 0.499657260755571</td>
</tr>
<tr>
<td>(57, 97)</td>
<td>7000</td>
<td>0.998216360246408 0.499657240038846</td>
</tr>
<tr>
<td>(0.00001, 0.00001)</td>
<td>50,000</td>
<td>0.969352825967765 0.500465448711644</td>
</tr>
<tr>
<td>(200, 100)</td>
<td>600</td>
<td>0.998526187170310 0.499648679877288</td>
</tr>
</tbody>
</table>

With starting point at (1.1, 0.45), the sensitivity method takes more computational time than the steepest descent method with direct simulation. In a small problem like this one, the computation of sensitivity information repeatedly may take more time than direct simulation of the functions. In a large scale problem, the direct simulation may become more and more time consuming, and the sensitivity information may become time-saving.

**Topic 5: Kinetic inversion with global terrain valley-tracking method**

With the steepest descent algorithm, the range of initial guesses that the algorithm admits is very narrow. With the sensitivity response algorithm, a large range of initial guesses are admitted. The global terrain methodology has its own advantages. For example, the greatest advantage of the global terrain methodology is the ability to identify multiple local minima, while the steepest descent and the sensitivity response surface algorithms would both get stuck at the first minimum and could not advance. Let us analyze the surface topology of the residual error surface and identify the main hurdles to the search of the solution.

**5.1 The topology of the residual error surface**

The residual error surface of the with k1 between [0, 10.0] and k2 between [0, 10.0] is shown in Figure 61. The true solution, (k1, k2) = (1.0, 0.5), is marked by the red point.
Figure 61. The terrain of the residual error surface with \( k_1 \) between \([0, 10.0]\) and \( k_2 \) between \([0, 10.0]\). The true solution, \((k_1, k_2) = (1.0, 0.5)\), is marked by the red point. The limited region of the initial guesses that the steepest descent algorithm admit is marked by the grey box, which means that we need to have a pretty good idea of the values of \( k_1 \) and \( k_2 \) as initial guesses. This expectation is not realistic. The kinetic inversion algorithm needs to admit bad initial guesses far away from the solution. Due to the difficult terrain characterized by the flatness (blue-point 1) with near-zero gradient and the valley (purple-point 2) where the gradient method gets stuck, we need an algorithm that takes advantage of the terrain topology. We will use the valley tracking method to follow the small valley marked by the purple point 2 which eventually directs us to the solution.

The limited region of the initial guesses that the steepest descent algorithm admit is marked by the grey box, which means that we need to have a pretty good idea of the values of \( k_1 \) and \( k_2 \) as initial guesses. This expectation is not realistic, however, since most of the time we do not know a priori where the parameter values lie. Therefore, a robust algorithm needs to admit bad initial guesses orders of magnitudes away from the solution.

This particular topology has two main hurdles: first, the flatness of the region, and second, the long valley near the boundary. Due to the difficult terrain characterized by the flatness (blue-point 1) with near-zero gradient and the valley (purple-point 2) where the gradient method gets stuck, we need an algorithm that takes advantage of the terrain topology. We will use the global terrain valley-tracking method to follow the small valley marked by the purple point 2 which eventually directs us to the solution.

5.2 Following the valley: the hessian and the eigen-directions

The properties of the hessian matrices, the eigenvalues, and the eigen-directions are examined at different points on the surface.

At the solution, \((k_1, k_2) = (1.0, 0.5)\), the hessian matrix is:

\[
\begin{bmatrix}
0.05475860323493 & 0.028334328093465 \\
0.028334328093465 & 1.075488452808314
\end{bmatrix}
\]  

The eigenvalues are 53.971619740271 and 1076.275436300535. Since all eigenvalues are positive, one can see that the hessian is positive definite. Coupled with a zero gradient, a positive definite matrix at (1.0, 0.5) means that all directions at this point are ascending directions. This is a local minimum or global minimum. Indeed, we know that (1.0, 0.5) is a global minimum since it is the solution to this minimization problem. The eigenvectors at this point is \((-0.999615019040589, 0.027745516907823, 0.999615019040589)\) for the first eigenvalue, and \((0.027745516907823, 0.999615019040589)\) for the second eigenvalue, respectively.

Now let’s look at the Hessian of the error surface at the parameter set (20, 20). This point is very far from the solution, and this point sits on a very flat region where the gradient is nearly zero in both directions. The hessian at (20, 20) is:

\[
\begin{bmatrix}
-0.172749196281075 & 0.009234270237357 \\
0.009234270237357 & -0.244935563387116
\end{bmatrix}
\]  

The eigenvalues at (20, 20) are \(-0.246098112893701\), \(-0.171586646774490\), and the eigenvectors corresponding to the first eigenvalue is \((-0.124909127176149, 0.992168186321297)\), and the second eigenvector is \((-0.992168186321297, -0.124909127176149)\). Since both eigenvalues are negative, the hessian is negative definite. However, this does not mean that (20,20) is a local maximum, since the gradient at (20,20) is not zero.

At the 8th iteration where \((k_1, k_2) = (99.944211609323006, 0.479593728606584)\), by looking at the residual error surface, we know that we are within a valley (Figure 61). The eigenvalues are \(-0.00006537033130\), and \(1.392910178350518\). The eigenvector for the first eigenvalue is \((-
0.999999999985656, -0.000005356170002), and the eigenvector for the second eigenvalue is (-0.000005356170002, 0.999999999985656). The eigenvector associated with smallest absolute eigenvalue indicates the direction of the valley. Since the valley leads to the solution, an algorithm that traces the bottom of the valley is beneficial.

5.3 Performance of the global terrain valley-tracking methodology

The performance of the algorithm can be investigated by testing different starting points. The first point tested was [99.7, 0.48]. The combined global terrain valley-tracking and steepest descent algorithm converged to a solution with an epsilon of 1e^-04 in 74 iterations. Out of the 74 iterations, 12 iterations were the valley-tracking steps, and 62 iterations were normal steepest descent steps. In other words, the hessian information was computed 12 times, and the gradient information was computed 62 times before the true solution was found. The converged solution is (k1, k2) = (0.999720383473956, 0.499535936319185), and remember that the true solution is (1.0, 0.5).

Topic 6: Confidence intervals for k1 and k2 using individual and joint confidence region

5.3.1 Individual confidence region

The visualization of student t distribution, and identification of t-scores are shown in Figure 62. T-scores for 90% is 1.65 and for 99% is 2.594 for this kinetic inversion problem with 280 degrees of freedom (94 data points for C_A, C_B and C_C minus 2 parameters estimated). From the confidence intervals (90% and 99%) of k1 and k2, one can observe that the k2 estimated from data is much more accurate than k1, since k2 has a narrower confidence interval than k1 (see Table 15, Figure 63).

Table 15. Individual confidence intervals for k1 and k2 with 90% and 99% confidence

<table>
<thead>
<tr>
<th>confidence</th>
<th>90%</th>
<th>99%</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-score</td>
<td>1.65</td>
<td>2.594</td>
</tr>
<tr>
<td>K_1</td>
<td>1.00233 ± 0.0021763</td>
<td>1.00233 ± 0.0034214</td>
</tr>
<tr>
<td>K_2</td>
<td>0.49975 ± 0.0004915</td>
<td>0.49975 ± 0.0025418</td>
</tr>
</tbody>
</table>

Figure 62. Student t distribution for degree of freedom = 280 and confidence intervals of 90% and 99%, respectively. The t-score signifies the vertical cutoff line where the area under the curve is 90% or 99% percent of the total area under curve. The t-score for 90% is 1.65 and...
for 99% is 2.594. The t-scores are used to compute confidence intervals of k1 and k2.

Topic 6.2: Joint confidence region

The joint confidence region for kinetic inversion of 2 parameters is expressed by an ellipse in Figure 64. The formula for the ellipse containing the inverse of the variance matrix (Eqn 53), the hessian (Eqn 54), and the final form of the ellipse equation (Eqn 55) are shown. The 2 by 2 matrix in Eqn 54 is equivalent to the hessian matrix, which is the inverse of the estimated variance matrix. Note that the estimated variance of the parameters is related to the hessian of the residual error surface, which indicates the ‘curvature’ of the residual surface at the solution. Large values in the hessian matrix indicate high confidence in the point estimation, see Eqn 54, since having large curvature at the residual surface minimum means that the surface is not featureless at the minimum. The axis length of the ellipse are 0.0032 and 0.000727, and the center of the ellipse is (1.00233, 0.49975). The eigenvectors of the hessian matrix define the axes of the ellipse, therefore, the rotation of the ellipse.

\[
\begin{bmatrix}
  k1 & 1.00233 \\
  k2 & 0.49975
\end{bmatrix}
\begin{bmatrix}
  0.174 \cdot 10^{-4} & -0.00468 \cdot 10^{-4} \\
  -0.00468 \cdot 10^{-4} & 0.00089 \cdot 10^{-4}
\end{bmatrix}
\begin{bmatrix}
  k1 - 1.00233 \\
  k2 - 0.49975
\end{bmatrix}
\leq 2 \cdot F
\]

Equation 53

\[
\begin{bmatrix}
  k1 & 1.00233 \\
  k2 & 0.49975
\end{bmatrix}
\begin{bmatrix}
  0.05826 \cdot 10^{-7} & 0.030049 \cdot 10^{-7} \\
  0.030049 \cdot 10^{-7} & 1.1416247 \cdot 10^{-10}
\end{bmatrix}
\begin{bmatrix}
  k1 - 1.00233 \\
  k2 - 0.49975
\end{bmatrix}
\leq 2 \cdot F
\]

Equation 54

\[
0.05826 \cdot 10^{-7}(k1 - 1.00233)^2 + 0.030049 \cdot 10^{-7}(k2 - 0.49975)(k1 - 1.00233) + 0.030049 \cdot 10^{-7}(k2 - 0.49975)^2 \leq 6.06
\]

Equation 55

Topic 7. Case study: Parameter estimation for aquaporin transcription and translation rates

7.1 Kinetic inversion with two sets of concentration data: mRNA and aquaporin

Synthesized concentration trajectories of mRNA in the cytoplasm and aquaporin channels on the membrane are used to determine two parameters, k7 and k13. Note, k7 is the transcription rate, and k13 is the translation rate. The structures of the residual surface and the log residual surface are shown in Figure 65.

In Figure 65, top frame, one can see that the residual error surface has a long valley along the k7 axes. This means that the surface is not very sensitive to the parameters k7. Next to the main valley, there is also a small crevice along the k7 direction around k13 = 11.5*10^-4. This surface is not convex, and the search of the minimum on the surface is difficult due to the presence of the valley and crevice. The search algorithm will need to have uphill movements during the search. The global terrain method should satisfy this requirement.
The log error surface shown in Figure 65 bottom frame has a sharp minimum, and a long valley leading to the minimum. There are multiple local minima on the long valley. These multiple local minima may prevent the search algorithm from reaching the true minima.

7.2 Kinetic inversion with three sets of concentration data: mRNA, aquaporin, and ribosomes

Kinetic inversion was performed in order to determine $k_7$ and $k_{13}$, the transcription and translation rate of aquaporin. The residual error surface and the log residual error surface are shown in Figure 66. The steepest descent coupled with valley-tracking algorithm was used. The algorithm was initialized at $k_7 = 3 \times 10^{-3}$, and $k_{13} = 0.8 \times 10^{-3}$.

The algorithm converge to the solution $k_7 = 4.9542 \times 10^{-3}$ and $k_{13} = 1.1673 \times 10^{-3}$ with a breakoff criteria, epsilon, of $10^{-10}$. The true solution is $k_7 = 5 \times 10^{-3}$ and $k_{13} = 1 \times 10^{-3}$. It is possible that the accuracy is affected by the small magnitudes of the parameter values, since both parameters are in the $10^{-3}$ range. It is harder to accurately determine parameter values with extremely small magnitudes than those with larger magnitudes. Therefore, the non-dimensionalization of the parameters may be necessary to resolve this problem. Another reason for the deviation of the determined values to the true solution is that the bottom of the residual error surface is featureless (very flat), which makes the search for the solution difficult. Searching on the log residual surface may resolve this issue.

7.3 Topology of residual error surface with different concentration data availability

It was hypothesized that once the number of available data sets increase, which means that once more species concentrations are measured, the residual error surface will become smooth and easy for kinetic inversion. Therefore, this hypothesis is tested by increasing the number of concentration data sets for three species at a time. Previously, the residual error surface of using three species concentrations was shown in Figure 66. The residual error surface for using six sets of concentration data is shown in Figure 67. Residual error surface for using nine sets of concentration data is shown in Figure 68.

Table 16. Number of concentration data sets used for computing the sum of squared error

<table>
<thead>
<tr>
<th>3 sets</th>
<th>6 sets (in addition to 3)</th>
<th>9 sets (in addition to 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic mRNA</td>
<td>RNA polymerase</td>
<td>Transcription factor 2</td>
</tr>
<tr>
<td>Aquaporin channels</td>
<td>TF1</td>
<td>Bound transcription factor 1</td>
</tr>
<tr>
<td>ribosome</td>
<td>RNA in nucleus</td>
<td>RNA bound to ribosomes</td>
</tr>
</tbody>
</table>
From Figure 67 and Figure 68, the expected improvement in the quality of residual error surface is not seen when more concentration data becomes available. The residual error surface has a long valley, and it has thin crevices on each side of the valley. The thin crevices may represent the presence of multiple stationary points. The residual error surface does not have a unique minimum and the determination of the kinetic parameters on this surface will be difficult with a deterministic algorithm. A stochastic algorithm such as the genetic algorithm may have better performance.

7.4 Kinetic Inversion during dynamic transitions

A dynamic transition was introduced into the reacting system by a temporary decrease in the transport rate of RNA in the nucleus to the cytoplasm. The concentration trajectories are shown in Figure 69. In the left frame of Figure 69, there is an expected increase in RNA concentration in the cytoplasm (green line) at $2 \times 10^5$ seconds, due to the decrease in RNA-exportin binding rate. This decrease in RNA transport causes the RNA to accumulate in the nucleus, and results in a slight drop in RNA concentration in the cytoplasm (dotted blue line). However, in the right panel of Figure 69, the concentrations of TF1, TF2, TF1 bound gene, and TF2 TF1 RNAP bound gene are not affected by this stimulus.
Figure 69. Dynamic transitions of the aquaporin reacting system when the RNA transport rate is decreased at 20,000 seconds. Dynamic transition of the reacting system is imposed by a decrease in the transport rate of RNA in the nucleus to the cytoplasm. From the left frame it can be observed that RNA in the nucleus accumulated due to the decrease in transport.

The residual error surfaces are obtained by the sum of squared error of the concentrations for 6 hours. For comparison, residual error surfaces are obtained using 3 data sets (Figure 70) and 9 data sets (Figure 71), respectively. From Figure 70, it can be observed that the crevices around the central valley disappeared, and the residual error surface appears smooth compared to Figure 66 which also uses three data sets (but during the approach of the system to steady state). The residual error surface is better conditioned using concentration data during the dynamic transitions of the reacting system. The topology of the residual surface using 9 data sets is similar to that using 3 data sets, and the quality is not improved by the addition of available data. This contradicts the hypothesis that the addition of the concentration data may improve the quality (smoothness, convexity, topology) of the surface, and the reason for this contradiction of our hypothesis is examined in the discussion section.

Figure 70. Residual error surface with 3 data sets during a dynamic transition of the reacting system. Figure 71. Residual error surface with 9 data sets during a dynamic transition of the system.
The crevices around the central valley disappeared, and the smoothness of the surface is improved.质量 of this surface is similar to that using 3 data sets. The lack of improvement of the surface quality despite the addition of six data sets contradicts our hypothesis.

7.5 The impact of the translation rate on the reacting network

From the residual surface of estimating k7 and k13, it can be observed that the model error is not sensitive to a change in k13. Since the valley on the residual surface lies along the k13 direction, it means that even though k13 deviates from the correct value, the sum of squared error remains small. It is therefore necessary to confirm whether changing k13 really does not affect the concentration trajectories. The integration of the reacting system is carried out with 3 different values of k13 and the trajectories are shown in Figure 72.

Figure 72. Concentration trajectories of aquaporin transcription and translation with three different rate of translation (k13). Trajectories with the true translation rate, k13 = 10^{-3}, are shown in the left panel. Trajectories with double the translation rate, k13= 2*10^{-3}, is shown in the middle panel. Trajectories with triple the translation rate, k13 = 3*10^{-3}, is shown in the right panel. There are no distinct differences in the concentration trajectories between the left, middle and right panels. This may explain why the model error is small regardless the change in k13.

Top frames of Figure 72 shows the concentration trajectories of RNA in the nucleus, RNA in the cytoplasm, RNA bounded to ribosomes, unfolded proteins, and folded proteins. These trajectories rise and reach steady state as expected. Bottom frames of Figure 72 show the concentration trajectories of TF1 bound gene, TF2 TF1 bound gene, and TF2 TF1 RNAP bound gene, and these three species are related to the transcription. These three species in the bottom frames are not affected at all by the change in k13.

From Figure 72, it can be observed that the concentration trajectories are not much affected by doubling or tripling the translation rate. Looking at the chemical reaction equations, k13 would only influence Eqn 28 and Eqn 29. Since Eqn 28 is an irreversible reaction, the value of k13 would not influence the reactions prior to Eqn 28. Therefore, the impact of k13 is limited to the last few species of the model. This is the reason why the model error is not very sensitive to a change in the parameter k13, since most of the concentrations are unaffected.

Discussion

Performance of steepest descent, sensitivity response surface, and valley-tracking method
Steepest descent algorithm is the easiest to implement, since it only uses the first derivative information. However, steepest descent algorithm only converges to the solution when initial guesses are very close to it. It fails to converge when the initial guess is orders of magnitude away from the true solution. The steepest descent algorithm in its raw form is not robust.

The sensitivity response surface algorithm shows excellent converge for a wide range of initial guesses. Guesses that are $10^3$ times greater than the true solution and those that are $10^5$ times smaller than the true solution will still converge. For the kinetic inversion of $k_1$ and $k_2$ in the reaction network of $A, B$ and $C$, the true solution is $(k_1, k_2 = 1.0, 0.5)$. For both $k_1$ and $k_2$, initial guesses starting from between 0.00001 and 2000 converged using the sensitivity response surface algorithm. One shortcoming is that this algorithm cannot solve a residual surface with multiple minima. This algorithm can only identify one local minimum.

The valley-tracking method can efficiently travels a valley (or crevice) on a residual error surface, where the steepest descent and response surface methods cannot. The ability to travel along the valleys has the potential to identify all local minima on a surface with multiple minima.

**Steady state and dynamic transitions of the reacting system**

The topology of the residual error surface strongly dictates the algorithm that must be selected for kinetic inversion. In the aquaporin case study, the residual error surfaces were obtained when the reacting system was approaching a steady state, and when the system was undergoing a dynamic transition. The residual surfaces obtained from steady state simulations have valley and crevices, as well as multiple stationary points. The residual surfaces obtained from dynamic state simulations are smooth and free of multiple stationary points.

From this study, we learn that the kinetic inversion of a reacting system is easier and the residual surface is better conditioned when the system is undergoing a dynamic transition and the concentrations are changing. We may infer that when we perform experimental measurements, the concentration measurements are more valuable if there is an external stimulation to the system and the system is undergoing a dynamic transition. When the system is given some initial conditions and is simply allowed to reach steady state, the concentration measurements do not contain much information about the rates of the reacting system.

**Quality of residual error surface as a function of data availability**

The reacting system usually contains multiple species, whose concentrations change over time. The ease of kinetic inversion should depend on the availability of concentration data for each species in the system. We hypothesized that the residual error surface is better conditioned when data sets of more species are available. This hypothesis was tested in the aquaporin case study for the inversion of two kinetic parameters with a total of 15 species.

First, data sets for only two species are used for constructing the residual surfaces. The reacting system was given initial concentrations and was allowed to reach steady state. The residual error surface contains a valley with a thin crevice on each side of the valley, which is a very ill-conditioned surface. Gradient-based search algorithms will fail on this residual surface. Then, the quality of the surface was investigated for 3, 6, and 9 data sets. This means that concentration data over time for 3, 6, and 9 species are available, respectively. When 9 sets of concentration data are used, the residual surface still contains the valley, and the crevices on each side. The quality of the residual surface did not improve as expected when more data are used for inversion.

As for the residual surfaces obtained from dynamic state simulations, the quality of the surface was very similar for using 6 concentration data sets and 9 data sets. It appears, at least in this case study, that the addition of concentration data does not aid in the kinetic inversion of unknown parameters. The quality of the residual error surface remain the same despite the addition of data. Two concentration data sets were sufficient for the kinetic inversion of two parameters in the aquaporin case study.

**Kinetic inversion on residual error surfaces with difficult topology**
The kinetic inversion of parameters with difficult, or ill-conditioned, residual surfaces is discussed in this section. First, observing the reacting system during dynamic transition is more informational than during the approach to steady state. Dynamic perturbation of the reacting system gives more information about the interaction of kinetic rates. Second, depend on the parameters to be inverted, the addition of concentration data may or may not be useful for the inversion problem. In the aquaporin case study, the kinetic parameter k13, the translation rate, dictates the rate for an irreversible reaction near the end of the reacting system. The species concentrations which occur prior to this reaction were unaffected by the specific value taken by this parameter. As it turns out, the residual error surface is very insensitive to changes in this parameter, resulting in a long valley where the perturbation of k13 gives a low error everywhere in the valley. In this case, the addition of concentration data does not improve the ease of the parameter search. The role of a particular unknown kinetic parameter in the reacting system, such as whether it governs a reversible or irreversible reaction, or whether the reaction is located at the beginning or end of the reaction cascade, is a key factor in the ease and feasibility of kinetic inversion problem.

If it is necessary to perform kinetic inversion on an ill-conditioned residual error surface and the surface quality cannot be improved, the choice of the search algorithm has paramount importance. If the topology of the surface is known or if the multiple minima appear to be connected by valleys or ridges, the global terrain method is advantageous. However, as states before, the knowledge about the surface topology is required for the implementation of the global terrain methodology. If the surface is discontinuous and not smooth, or if the topology is unknown or unpredictable, the genetic algorithm, which is a stochastic algorithm, may be more advantageous.

Conclusions

This report documents solving the kinetic inversion problem with steepest descent algorithm, the sensitivity response surface algorithm, and the valley-tracking technique from global terrain methodology. The sensitivity response surface algorithm is the most robust, admitting a wide range of initial guesses. The steepest descent algorithm is the easiest to implement computationally, but it will not guarantee convergence from bad initial guesses. Though difficult to implement, the valley-tracking algorithm can identify multiple minima on the residual surface. The choice of the search algorithm strongly depends on the topology of the residual error surface and the features of the terrain.

Kinetic inversion of the transcription and translation rates was performed for the aquaporin gene network. Due to the nonlinearity of the gene network, the residual error surfaces were ill-conditioned, containing a central valley and thin crevices. The residual error surface of the reacting system when the system is undergoing a dynamic transition is better-conditioned than when the system is reaching a steady state. However, the quality of the residual surface is unaffected by the addition of the data in the aquaporin case study. This may be due to the fact that the species concentrations in the model are not sensitive to the parameter chosen for estimation. Sensitivity information of concentration trajectories in respect to the unknown parameters may give additional information on kinetic inversion.
Appendix 6. Aquaporin-4 water channels in neurological injuries - a review.

Introduction: Aquaporin-4 water channels and neurological disorders

Many studies have quantified AQP4 expression levels over time. After hypoxic-ischemic/reperfusion injury, AQP4 levels decreased and reached a minimum during the first 24 hours, and then its levels increased afterwards.(92) The phases of decrease and increase roughly correspond to the occurrence of cytotoxic and vasogenic edema. In cytotoxic edema, the main cell type that experiences swelling is astrocytes,(93) and this swelling of astrocyte processes and cell bodies is mediated by the AQP4 channels. A protective effect of AQP4 downregulation in cytotoxic edema has been observed,(94) in agreement with the role of AQP4 in astrocytic swelling. On the other hand, upregulating AQP4 has been found to improve survival in vasogenic edema.(95) The physiological response of AQP4 dynamics characterized by an initial decrease and subsequent increase in its expression levels may have a protective role. These studies provide evidence that AQP4 levels change dynamically during neurological disorders, but do not provide explanations for these changes. An understanding of the signaling mechanisms that control the transcriptional regulation of the aqp4 gene and post-transcriptional regulation can enable systematic analysis of AQP4 expressions in normal conditions and during disease progression in the CNS. Furthermore, in order to design effective molecular therapies, we need to determine whether the natural AQP4 dynamics are a desirable response with a protective role or an undesirable one. Understanding this dynamic level change will determine the dose and timing of pharmacological interventions. This review correlates the changes in microenvironment of the brain with AQP4 expression dynamics through molecular signaling.

The reason why understanding the detailed signaling mechanism is crucial is two-fold:

1. Dynamic trajectories of AQP4 expression levels in disorders such as edema, hydrocephalus, ischemia, and traumatic brain injury have been observed. However, few reports are available that explain the cellular signaling/regulation events that lead to the documented AQP4 expression.

2. The development of novel molecular therapies cannot be solely based on the measurement of input-output relationship. The most therapeutically-tractable strategies can only emerge based on detailed analysis of signaling cascades and possible downstream events after the administration of a compound.

In the first part of this article, the influence of stress factors caused by brain injury on the expression of aqp4 gene will be reviewed in detail. The goal of this section is to understand the dynamic expression levels of AQP4 in brain injury with a focus on ischemic injury. By combining knowledge on stress factors with signaling cascades and cellular biochemistry, we provide possible explanations for the mechanisms behind dynamic AQP4 expression levels post-injury. This section first describes the changes in the brain microenvironment during neurological disorders, followed by the activation and inhibition of the aqp4 gene through signaling cascades induced by various inflammatory cytokines, oxidative and osmotic stresses. A molecular circuitry describing the relationship between stress factors induced by injury and aqp4 gene regulation is presented to elucidate the link between brain injuries and changes in AQP4 mRNA and protein levels.

The second part of this article focuses on the post-translational modification mechanisms which influence the expression, trafficking, recycling, and water permeability of aquaporin-4.

The last part of this review summarizes the signaling mechanisms of different small molecules for controlling AQP4 expression levels.
Aquaporin-4 isoforms

The existence of multiple AQP4 isoforms has been associated with distinct translational efficiencies, organ-specific expression, developmental regulation, the formation of square arrays on cellular membranes, or different water conduction rates. In rats, six cDNA isoforms resulting from alternative splicing have been discovered.\(^{(96)}\) Even though six different AQP4 protein isoforms are theoretically possible, three of them have never been observed in vivo. Only three AQP4 protein isoforms, the M1, M23, and Mz isoforms,\(^{(97)}\) have been observed in rat brains. In the brain, the ratio of M23 to M1 isoforms is found to be 7:1,\(^{(98)}\) and the quantity of Mz isoform is very scarce. In humans, only two isoforms, M1 and M23, are observed.

These three AQP4 isoforms differ in their ability to form orthogonal arrays (or square arrays) on cellular membranes. The M23 isoform forms large square arrays,\(^{(99-100)}\) the M1 isoform forms small or unstable square arrays,\(^{(99-100)}\) and Mz(97) isoforms do not form square arrays. The function of orthogonal square arrays is currently unknown.

These isoforms result from the use of alternative promoters of the aqp4 gene. The M1 isoform is encoded by exon 0, and the M23 is encoded by exon 1. Differential abundance of AQP4 isoforms in the brain during normal conditions and disease progression could arise from transcriptional activation of alternative promoters or post-transcriptional regulatory mechanisms such as microRNA.

Alternative promoters and their transcription factors of aquaporin-4 gene

To understand the transcriptional regulation mechanism, Umenishi and Verkman have studied the promoter regions associated with the exon 0 and exon 1 of human aqp4 gene. Exon 0 codes for the M1 isoform of AQP4, and exon 1 codes for the M23 isoform. Promoter regions are sites where transcriptional regulators and RNA polymerase bind to initiate transcription of a target gene. Different transcription factors regulate the transcription of different sets of genes responsible for diverse cellular processes. The activation of these transcription factors by phosphorylation or transcriptional upregulation will alter the expression of a target gene.
Regulation of M1 isoform. The exon 0 promoter contained 4 TATA boxes, 5 CCAAT boxes, AP-1, Sp1, and E-box elements.\(^{(101)}\) CCAAT box is known to bind the CCAAT-enhancer binding proteins (c/ebp), indicating that aqp4 gene expression can be influenced by this transcription factor. The AP-1 promoter element interacts with the activating protein-1 (AP-1) transcription factor. The Sp-1 promoter is bound by the specificity protein-1 (Sp-1) transcription factor. E-box is bound by upstream stimulating factor-1 (USF-1), but many other elements are known to interact with E-box. In the M1 promoter region, E-box is known to have strong inhibition activities.\(^{(101)}\) Recently, an astrocyte-specific enhancer associated with the Pit-1/0ct/Unc-86 (POU) family transcriptional factors has been identified in the 5’ flanking region of the mouse aqp4 gene.\(^{(102)}\) By construction and analysis of reporter constructs containing exon 0 or exon 1 promoters, it was determined that the transcriptional activity of exon 1 is much stronger. However, promoter activity of exon 0 has stronger relative activity in brain derived cells than kidney-derived cells, indicating a brain-specific AQP4 isoform regulation.

Regulation of the M23 isoform. The exon 1 promoter contained 1 TATA box, AP-1, AP-2, and E-box elements.\(^{(101)}\) The AP-2 promoter interacts with activating protein-2 (AP2). AP-2 is a developmentally regulated transcription factor, and it is highly expressed in neural crest cells in mouse embryos but scarcely expressed in adult mice brains.\(^{(103)}\) Primary astrocytes from mice express low levels of constitutive AP-2 mRNA, but its mRNA expression can be stimulated by cAMP, noradrenaline, and isoproterenol.\(^{(104)}\) Evidence shows that the expression of both M1 and M23 isoforms of AQP4 increased in the postnatal periods of rats.\(^{(105)}\) The possible role of AP-2 in AQP4 developmental regulation remains to be elucidated. Recently, a putative binding site for NF-κB transcription factor in exon 1 promoter construct has been found.\(^{(106)}\)

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has also been proposed to interact with the aqp4 gene, however, whether this regulation is isoform-specific is not known.\(^{(107)}\)

Aquaporin4 expression dynamics in brain injury

In many disorders, AQP4 expression levels show a dynamic temporal trajectory. In a rat model of hydrocephalus, AQP4 expression levels decreased and reached a minimum at 2 days, followed by a subsequent recovery and increase.\(^{(108)}\) In ischemia, a similar trend with initial decrease followed by an increase is found, and this dynamic change corresponds to stages of cytotoxic and vasogenic edema. This complex time-evolution is likely caused by different regulation mechanisms at both the transcriptional and post-transcriptional levels, and these regulation mechanisms are induced by changes in the brain microenvironment during injury. To understand the cellular regulation mechanisms behind the dynamic expression levels of AQP4, it is necessary to first gain an understanding of the temporal changes in brain...
microenvironment after injury, and then correlate potential stress factors with cellular signaling mechanisms that lead to changes in AQP4 expression.

Neurological disorders and brain injuries produce mechanical and biochemical stress factors which in turn cause changes in cellular gene expressions, including the expression of aqp4 gene. In pathologies involving brain water regulation such as hydrocephalus and edema, the change in aqp4 gene expression is of great interest for the development of pharmacological intervention therapies. Therefore it is important to identify the time-evolution of stress factors caused by these neurological disorders, and how they impact AQP4 expression post-injury. The following discussion focuses on how the stress factors in different stages of ischemic brain injury correlates with dynamic AQP4 expression levels through molecular signaling mechanisms and regulation at transcriptional and post-transcriptional levels.

**Dynamics of aquaporin-4 in ischemic injury**

Cerebral ischemia is a condition characterized by reduced blood flow and oxygen supply due to an occlusion event. Transient or permanent ischemic injury induce changes in the brain microenvironment such as a dynamic shift of osmotic gradients between blood, extracellular space and intracellular space, as well as oxidative stress. These changes in the microenvironment activate signaling cascades which alter gene expression levels, including that of AQP4. The release of signaling molecules and inflammatory cytokines into the brain causes distinct effects on aqp4 gene regulation. In addition, AQP4 levels in ischemia are subject to translational regulation mechanisms by specific microRNAs.

AQP4 expression after ischemic injury shows a complex temporal and spatial pattern. In a transient focal ischemia model with reperfusion following 30 minutes of occlusion, AQP4 expression was found to increase at 1 hour, returned to normal levels at 6 hours and 24 hours, and decreased at 48 hours at the ischemic core. In the border, similar patterns are observed at 1, 6, and 24 hours, however AQP4 expression increased at 48 hours.(109)

Changes in the brain microenvironment can cause secondary injury mechanisms such as cytotoxic edema, vasogenic edema, and brain inflammation. Expression levels of AQP4 are tightly linked to the genesis and resolution of edema. Understanding the role that AQP4 channels play in these injury mechanisms is crucial to the development of pharmacological therapies, which will be discussed in the next section. This section presents an overview of the signaling pathways induced by changes in the brain microenvironment that are highly relevant to analyzing the dynamic AQP4 expression after ischemia.
Manifestations of oxidative stress and its influence on AQP4 expression

Transient cerebral ischemia causes oxidative stress by ischemic-reperfusion injury. A low level of reactive oxygen species (ROS) is generated in the mitochondria (29-30) during normal cellular processes, and these ROS are sequestered by antioxidant enzymes. Excess generation of ROS by the mitochondria is found after ischemic reperfusion injury, traumatic brain injury,(110-113) traumatic spinal cord injury,(114) and hemorrhage.(115) Excessive generation of ROS overwhelms the intracellular antioxidant capacity leading to apoptosis and necrosis [Liu, Pinatadosi]. A sudden elevation in oxygen levels due to blood reperfusion provides oxygen as a substrate for further oxidizing reactions in the cell, causing ischemic reperfusion injury. [Chen] In the case of severe ischemia, the mitochondria becomes incapable of producing ATP, which leads to the suspension of energy dependent ion pumps and the disturbance of osmotic balance in the brain.(116)

Oxidative stress causes changes in gene expression. The induction of oxidative stress is followed by a rapid elevation of immediate early genes such as c-JUN and c-FOS,(117-118) and an enhancement in the DNA binding activity of activating protein-1 (AP-1).(117, 119) Oxidative stress also activates a key transcription factor for inflammatory response, NFκB,(120-122) and this activation can be blocked by antioxidants. AP-1 and NFκB are modulators of acute phase response, and they are known transcriptional activators of aqp4.
gene. The dynamic expression of AQP4 channels after brain injury could be related to the presence of oxidative stress.

The presence of oxidative stress induce the expression of phase-II detoxifying enzymes through activation of genes containing antioxidant response element (ARE) via NFE2-related factor-2 (Nrf2). The activation of Nrf2 in astrocytes protects neurons from oxidative damage, and incurs neuroprotection. The promoter region of aqp4 gene contains putative AREs, and Nrf2 has been found to mediate sulforaphane-induced AQP4 upregulation. The nuclear translocation of Nrf2 caused by oxidative stress is tightly linked with the PI3K signaling pathway. However, the signaling pathway responsible for Nrf2 activation in astrocytes has not been confirmed. The detailed mechanism of PI3K and Nrf2 activation is described below.

PI3K subunit p85 contains one of these SH2 domains and is able to bind to IRS1/2 and is recruited to the membrane. The binding of the PI3K p85 subunit and phosphorylated IRS1/2 leads to a conformational change in the PI3K complex, which results in the activation of the PI3K p110 subunit. Once phosphorylated, the p110 subunit can phosphorylate membrane lipids and Ser/Thr residues of proteins. Basolateral phosphatidylinositol-(4,5)-biphosphate (PIP2) is converted to phosphatidylinositol-(3,4,5)-triphosphate (PIP3) through direct interaction with the p110 subunit of PI3K. Akt contains an SH domain that binds to PIP3, causing a conformational change in Akt. This conformational change allows 3-phosphoinositide-dependent protein kinase (PDK1) to phosphorylate the Thr308 residue on Akt. Another phosphorylation happens at the Ser473 residue of Akt by the mammalian target of rapamycin complex 2 (mTORC2). This double phosphorylation is needed to fully activate Akt.

Oxidative stress activates other signaling pathways that play a role in inflammation response. In astrocytes, exposure to hydrogen peroxide, an inductive agent for oxidative stress, leads to phosphorylation and nuclear translocation of p38 and ERK MAP kinase pathways within 5 minutes. Activation of p38 pathway is known to induce transcriptional upregulation of AQP4, which provides another link between the presence of oxidative stress in the damaged brain and dynamic AQP4 expression. Generally, p38 mediates the astrocytic production of proinflammatory cytokines and nitric oxide synthase, indicating its role in brain inflammation.

Oxidative stress also activates JNK in astrocytes, leading to the apoptosis pathway through the activation of caspase-3. The activation of JNK is detected 24 hours after experimental subarachnoid hemorrhage, and JNK inhibition in a rat hemorrhage model decreased VEGF production, prevented BBB breakdown, and reduced MMP-9, caspase-3, and AQP1 levels. Even though JNK activation has no effect on AQP4 expression, JNK is highly related to water regulation in post hemorrhagic brain since its activation upregulated AQP1 expression by 5 fold after subarachnoid hemorrhage.

studied the effects of oxidative stress on phospholipid signaling astrocytes. Peroxide activated PLC and PLD (phospholipase C and D). In Ca2+-free medium, PLD response was abolished. The PKC inhibitor dramatically reduced PLD activity, implying that PKC has a role in PLD activation.

Oxidative stress activates many signaling pathways that lead to the expression of brain aquaporins, indicating that the resolution of oxidative stress in the brain and water regulation are tightly linked.

Ionic imbalance and astroglial swelling facilitated by AQP4

After ischemia, increased brain water content at the ischemic core indicates the formation of edema as early as 1 hour after the occlusion event. This early phase of edema is characterized by cell-swelling, which occurs mainly in astroglial cells. The preferential swelling of astroglial cells could be attributed to their expression of AQP4 channels, facilitating water flux in the presence of an osmotic challenge. The main cause of cell-swelling has been attributed to the failure of energy dependent ion pumps and the intracellular accumulation of cations like Na+, Ca2+ due to the failure of mitochondria to supply ATP during severe deprivation of oxygen. A significant intracellular increase of Na+ is detected within 60 minutes of global ischemia, accompanied by a shrinkage of extracellular space from 18.9% to 8.5% of cortical volume within 60 minutes. The intracellular increase of Na+ is mirrored by an extracellular accumulation of K+, even though the greater increase in Na+ still results in a hyperosmolar environment in the cell. These evidence
indicates that the initial phase of brain edema following ischemia is cytotoxic edema, and that a transient downregulation of AQP4 during this phase may be beneficial.

Astrocyte swelling is a common symptom in neurological disorders, and this swelling is related to an osmotic imbalance causing water influx into the cell. The normal regulation of cell volume in the presence of an osmotic insult consists of two phases. First, cell cycle arrest occurs, and cells attempt to re-establish osmotic balance by the transport of inorganic and organic osmolytes out of the cell.(138) After re-establishing osmotic balance, the cell volume is restored by a passive water efflux through AQP4. However, in the presence of energy deficiency, the active transport mechanism of ionic and other osmolytes fails. During the accumulation of cations in the cytosol, the extracellular environment is hypo-tonic, and water influx occurs through AQP4 channels, causing cell swelling. The detrimental role of AQP4 during the development of cytotoxic edema is demonstrated by AQP4-knockout mice, consistent with the view that AQP4 on astroglial cells facilitates cell-swelling during this stage of intracellular hyperosmolarity.(139)

Evidence of a hyperosmolality in brain tissue has been found after focal ischemia. The osmolarity of normal brain tissue - without differentiating the extracellular or intracellular environment - is 311.3 ± 2.2 mOsm/kg. Tissue osmolarity reached a maximum of 329.6 ± 1.9 mOsm/kg at 3 hours, marking an elevation of 18.3 mOsm/kg, and returned to control values 12 hours after occlusion.(140) At the 3 hour time point, the BBB is still considered to be intact, so the elevated osmolarity is not due to the extravasation of blood proteins. However, investigating the gradient between the intracellular and extracellular compartments is more relevant to the development of cytotoxic edema.

An osmotic gradient between the intracellular and extracellular compartment transcriptionally activates the aqp4 gene. Slight elevation in osmolarity (324 mosm) does not cause its transcriptional activation, while an osmolarity of 363 ± 2.7 mOsm stimulates a 1.5 fold increase in both AQP4 mRNA and protein.(141) Whether or not an osmotic insult in the brain environment causes transcriptional activation of AQP4 depends on the magnitude of osmotic gradient between the intracellular and extracellular compartment. Based on electrode measurements, this transmembrane osmotic gradient resulting from the accumulation of Na⁺ and K⁺ ions alone could amount to 65 mEq/kg,(116) without considering non-ionic osmolytes. Osmotic gradient of this magnitude should be sufficient to cause aqp4 transcriptional activation. At a later stage, a different phase of osmotic insult occurs after BBB breakdown and the extravasation of plasma proteins and ions from the cerebral vasculature, which will be discussed in the next section.

An osmotic gradient regulates the expression of AQP4 through both transcriptional and post-transcriptional regulatory mechanisms. Osmotic stress decreases the translational efficiency of yeast aquaporins. It is reasonable to hypothesize that this initial decrease suppresses water transport while an ionic balance is being re-established across the cellular membrane. After a hyper-osmotic shock, translational efficiency of yeast aquaporins decreased by 8 fold after approximately one hour. The portion of the mRNAs in the polysomal pool drops from 75% at normal conditions to 10%, which could be related to the storage of mRNAs in P-bodies.

An exposure to either hyper- or hypo-osmotic stress transcriptionally activate aqp4 gene. Hyperosmotic stress upregulates AQP4 through the p38 MAPK pathway,(141-142) which also controls the generation of proinflammatory cytokines from astrocytes.(130-132) An input of osmotic stress activates a member of the G-protein family known as Rac1 (143-144). Rac1 is located in the cytosol, and it is transported towards the membrane during activation(145). In the presence of osmotic stress, Rac is known to be activated by 2.5 fold (144). Rac1 then binds to the osmo-sensing protein OSM, followed by association with MEKK3 and M KK3 (143, 146-148), enzymes known as mitogen-activated protein kinase kinases(MAPKK). Once bound to the activated Rac1, these MAPKKs phosphorylate a specific MAPK pathway, p38.(146) In the inactive state, p38 is located in the cytoplasm. Once activated, p38 translocates to the nucleus via microtuble-based motor transport (149) and phosphorylates c-Jun and c-Fos, both components of activator protein 1 (AP-1) (150-151). c-Jun and c-Fos monomers can be combined to form either a homodimer (c-jun:cjun) or a heterodimer (cjun:cfos). The heterodimeric combination is more stable than the homodimer (152). Multiple AP-1 binding sites have been found in the promoter regions of the aqp4 gene, and AP-1 activation has been confirmed to upregulate AQP4.(153)
The induction of osmotic and oxidative stress in tissue and the accompanying cytotoxic edema occurs within 3 hours after ischemic injury. At a later stage, vasogenic edema occurs following the breakdown of BBB. This mechanism will be discussed in the next section.

**Increased permeability at the blood brain barrier and vasogenic edema**

The occurrence of vasogenic edema is a consequence of BBB breakdown. Many molecules including thrombin,(154) VEGF,(155-159) MMP-9,(155, 160) and leukotrienes,(161) play a role in orchestrating the desintegration of BBB. VEGF also induces apoptosis of endothelial cells by activating ERK1/2.(162) Both VEGF and MMP-9 increase AQP4 levels through unknown mechanisms.

BBB is said to be intact at 4 hours after ischemia.(163-164) The timing of BBB opening after ischemic injury has been measured based on the extrasavation of tracer molecules. A significant increase in brain albumin content due to extravasation occurs about 6 hours after ischemia.(137) A significant increase in BBB permeability was determined to be 48 hours,(165) 24 to 48 hours,(166-167) and 12 hours (168) after focal ischemia by studying the extravasation of Evans blue albumin, horseradish peroxidase, and radiolabeled bovine serum albumin. While protein extravasation used to be considered the osmotic force driving vasogenic edema,(169) the elevation of sodium ions and other cations in the brain has been confirmed to play a dominant role.(137) The imbalance in brain osmolarity and the release of plasma proteins could both affect AQP4 regulation.

**Effect of brain inflammatory cytokines and signaling molecules on aqp4 gene.**

Brain inflammation accompanies many disorders of the CNS. In TBI, the release of IL-1,(170) TNF-α,(171) IL-6(171) and excitotoxin glutamate(171) has been found in rodent models. Elevated levels of IL-10, TNF-α, and IL-1β and IL-2 are found after middle cerebral artery occlusion in the rat brain.(172) An increase of TNF-α, IL-6, and IL-1β are found after an transient cerebral ischemia.(173) In hydrocephalus, high levels of inflammatory cytokines such as TNF-α,(174) IL-1β(175) occur in the CSF. TNF-α(176) is also found after subarachoid hemorrhage. Amyloid-β deposits have been observed in Alzheimers.(177) Inflammatory cytokines have both therapeutic and deleterious effects in the brain through their interaction with specific receptors. The effects of two pro-inflammatory cytokines commonly found in neurological disorders, IL-1 and TNF, on aqp4 gene activation will be discussed.

Cytokines after injury come from many different sources, and one of the . The induction of immediate early genes and the activation of key transcription factors such as NFκB by earlier stress factors lead to the production of cytokines by brain cells. Through both intracellular signaling and intercellular crosstalk, these cytokines mediate the inflammatory response.

IL-1β is an important pro-inflammatory cytokine in the brain. Level of IL-1β mRNA started to increase after 15 minutes of focal cerebral ischemia, reached a maximum at 3 hours after, and became undetectable 4 days after.(178) The elevation in IL-1β protein levels is noticeable at 1 hour after focal ischemia, peaked at 4 hour after ischemia followed by a slight decrease, and reached a maximum at 3 days after ischemia.(179) The induction of IL-1β in post-ischemia brain is rapid. IL-1 is found to be elevated for more than 150 fold in subarachnoid hemorrhage,(180-181) ischemia,(182-186) severe head injury,(187) and hydrocephalus.(175)

IL-1β is a potent activator of a pro-inflammatory nuclear factor, NFκB, which in turn induces the transcription of genes which promote the inflammatory response. A putative binding site for NF-κB has been found near exon 1 of aqp4 gene(106) which regulates the induction of the transcription for the short M23 AQP4 isoform. The transcription of aqp4 gene is a part of the inflammatory response induced by IL-1. Indeed, Ito confirmed that IL-1β is a potent inducer of AQP4 expression in astrocytes, and this induction is mediated by NF-κB activation,(106) but not the p38, JNK, or ERK pathway. The activation of NFκB in astrocytes is mediated by PI3K and Akt signaling pathways after IL-1β stimulation.(188) Akt, also known as protein kinase B (PKB), activates NFκB by promoting the degradation of its inhibitory molecule IκB.(189-191) The activation of NFκB by Akt is shown in pink in Figure 73.
The injection of IL-1β has been shown to exacerbate brain edema after ischemic injury in rats, even though IL-1β injection into healthy animals do not change brain water content.(186) The upregulation of AQP4 by IL-1β in astrocytes could be partially responsible for edema worsening, while the upregulation of AQP4 in normal brains does not have deliterious effects due to the lack of an osmotic gradient. The reduction of this AQP4 upregulation through the inhibition of IL-1 signaling has been found to improve edema outcome. This evidence proves that the severity of brain edema after injury is a function of cytokine-induced AQP4 upregulation.

In addition to activating NFκB, IL-1β is shown to transcriptionally upregulate c/ebpβ and c/ebpδ (network) in astrocytes,(192) which are immediate early genes for the acute phase response. The promoter region of the M1 isoform of AQP4 is shown to have multiple binding sites for c/ebp. However, it is still not clear whether c/ebp interacts with the aqp4 gene through activation or inhibition mechanisms, since different c/ebp isoforms have opposite effects on gene transcription.

Another cytokine that is involved in the induction of inflammatory processes is tumor necrosis factor (TNF). In hydrocephalus, TNF is known to be elevated 100 times in the CSF. TNF induces IL-1 production in astrocytes,(193) and IL-1 also stimulates TNF production in astrocytes.(194) This positive feedback loop can cause a sustained elevation in cytokines, contributing to the sustained elevation of AQP4. TNF upregulates the expression of AQP4 in an epithelial cell line.(195) Experiments have found that not only does IL-1 modulate AQP4 expression in astrocytes, AQP4 levels induced by IL-1 significantly impact the resolution of edema.

AQP4 expression is found to be regulated by leukotrienes, which play an important role in inducing leukocyte-endothelial adhesion and increasing BBB permeability. Leukotrienes are produced by the 5-lipoxygenase pathway as a metabolite of arachidonic acid (AA).(196-198) Since oxidative stress stimulates the release of AA from the membrane,(199) the production of leukotrienes could be highly induced by oxidative stress. Leukotrienes are released into the brain after TBI,(200-201) fluid-perfusion injury,(202) ischemia,(197, 203) and hemorrhage.(203-205) While leukotriene B4 triggers leukocyte-endothelial adhesion after ischemia-reperfusion injury, cysteinyl leukotrienes mediate cerebral vascular permeability by promoting macromolecule leakage from vessels.(161) Cysteinyl leukotrienes are pro-inflammatory signaling molecules released from astrocytes and they mediate astrocyte proliferation and GFAP expression.(206) A member of the cysteinyl leukotrienes, leukotriene D4, is a potent activator of the leukotriene receptor-2 (CysLT2).(207) The activation of CysLT2 receptor induces AQP4 upregulation through the p38 and ERK signaling pathways, but not the JNK pathway.(208) This CysLT2 receptor-mediated AQP4 upregulation is partially responsible for the CysLT2 activation-induced ischemic injury in astrocytes, and could play a role in edematous brain injury.(208) The application of leukotriene synthesis inhibitor MK886 reduced leukocyte adhesion to endothelium and macromolecule leakage.(161) The p38 signaling pathway is shown in X and the ERK signaling pathway is shown in Y.

**Transcriptional inhibition of AQP4 by thrombin via PKC signaling**

Thrombin, a serine kinase, is a blood protein that plays an important role in the coagulation cascades after vessel injury. Thrombin is detected in the CSF after hemorrhage,(209) ischemia, TBI, and causes edema formation in the brain through an unknown mechanism.(210) Thrombin induces BBB disruption after the intraparenchymal injection into rat brains, which could be a possible mechanism of edema formation.(154) BBB breakdown is a cause of vasogenic edema through the release of blood proteins and ions, disrupting osmotic balance and incurring biochemical damage.

In vitro studies show that thrombin activates PKC signaling pathway in astrocytes and inhibit the transcription of AQP4.(211) The downregulation of AQP4 by thrombin via the PKC pathway is partially mediated by PAR-1 receptor. PKC activation downregulates AQP4 in a time-dependent manner and PKC depletion caused by prolonged treatment of the PKC activator reverses AQP4 downregulation.(211) The downregulation of AQP4 by thrombin could be a protective effect, since the activation of coagulation cascades coincides with early episodes of cytotoxic edema. Other agents that activates this PKC pathway and causes downregulation of AQP4 are PMA, TPA,(212-213) and propofol,(214) and this downregulation is caused by the decrease of mRNA at the transcriptional level.(212)
Post-transcriptional regulation of AQP4 in ischemia.

MicroRNAs are short endogenous RNA segments that bind to targeted mRNAs with a 7-mer seed match. MicroRNAs translationally repress target mRNAs. By interacting with the 3'-UTR region of the target mRNA, mammalian miRNAs target their bound mRNAs to P-bodies. The binding of the miRNA with Argonaute family proteins is necessary for their subsequent localization in P-bodies. In P-bodies, miRNAs interact directly with decapping enzymes Dcp1 and Dcp2. miRNAs that target the AQP4 mRNAs have been identified, and these miRNAs can control AQP4 levels through translational inhibition mechanisms.

Post-transcriptional control of AQP4 levels by miRNA has been observed in ischemic injury of the brain. Bioinformatic search reveals that 25 miRNAs are predicted to target AQP4 mRNA. Translational inhibition of AQP4 by miRNA320 and miRNA130 have been confirmed in rodent models of cerebral ischemia. For example, microRNA130a increased to about 2 fold at 1 hour and 24 hours after ischemia, and AQP4 showed corresponding decrease at these time points. Microarray analysis revealed that miRNA-130a was increased at 24 hours after ischemia, and miRNA320 was increased at both 24 and 48 hours. Moreover, miRNA130a specifically inhibit the M1 isoform of AQP4, but not M23 isoform, its specificity affords selective regulation of AQP4 isoforms. Translational regulation of different AQP4 isoforms by miRNA could lead to changes in cellular membrane water permeability. The translational inhibition of AQP4 transcript by miRNA is drawn in violet in Figure 73.

Underlying mechanisms of AQP4 regulation in ischemic brain

After reviewing changes in brain microenvironment and relevant signaling mechanisms, testable hypothesis are made in regards to the molecular mechanism behind the dynamic trajectory of AQP4 expression after ischemic injury. The initial decrease in AQP4 corresponds to the increase in AQP4-targeting microRNA, and further downregulation could also be induced by the extravasation of blood protein, thrombin. The later and sustained increase of AQP4 following the initial decrease could be a result of elevated levels of pro-inflammatory cytokines, oxidative stress and osmotic gradient, or molecules such as MMP. The combination of these temporally overlapping injury mechanisms produces an unique spatial-temporal AQP4 expression in the brain after injury, and these hypothesis still need to be further tested. Detailed analysis on the molecular perspectives of aqp4 regulation is required to decode the orchestrated response. The design of an efficient therapy to control AQP4 expression post-injury must also meet stringent requirements to produce desirable temporal and spatial dynamics.
AQP4 expressions in other neurological injuries

**Hydrocephalus**

In hydrocephalus, excess CSF accumulates in brain ventricles, causing edema near the periventricular region and deep white matter ischemia due to tissue compression. During the onset of kaolin-induced hydrocephalus, AQP4 level in the periventricular region was observed to decrease in the first 48 hours, and this decrease is followed by a recovery and a sustained increase. The causes of this initial decrease in AQP4 expression and its subsequent increase are not clear. However, elevated levels of cytokines could be a contributing factor. TNF-α is 45 fold higher in normal pressure hydrocephalus, as well as elevated levels of IL-1, IL-4 and IL-10. Hydrocephalic AQP4-null mice showed increased mortality rate, elevated intracranial pressures, and higher brain water content after kaolin induction compared to wild-type mice, confirmed a beneficial role of AQP4 in fluid clearance in hydrocephalus.

**Intracranial and Subarachnoid hemorrhage**

The type of brain edema that follows hemorrhage is mainly vasogenic in nature. This edema is said to be caused by BBB breakdown induced by the release of thrombin, an important component of the coagulation cascades. Elevated levels of thrombin is found in the CSF 3-5 days after subarachnoid hemorrhage. Thrombin is a potent inhibitor of aqp4 transcription through the PKC pathway.

**Traumatic brain injury**

AQP4 exhibits a complex spatial and temporal dynamics after TBI, and many conflicting evidences are reported. AQP4 is upregulated in the injury core of a traumatic brain injury (TBI) model in rats, while downregulated in regions around the injury core. In a closed-head model of TBI, AQP4 is found to be upregulated by more than 50% in the lysates of the whole brain 24 hours after injury. In addition, the inhibition of MMP-9 or HIF-1α inhibited AQP4 upregulation. TBI coupled with hypoperfusion can induce coagulation abnormaly and lead to pro-thrombic ischemic states, which influence AQP4 expression as discussed previously. In a contusion model of TBI, the expression level of AQP4 protein was found to diminished almost completely in the contusional cortex at 1 days after injury, while the mRNA was decreased to 50% compared to sham-operated animals. At the contusion core, vasogenic edema characterized by
compromised BBB integrity was found. After controlled cortical impact injury, Guo et al. investigated the pericontusional areas, the lateral ventricles, and the third ventricle, and found no significant changes in AQP4 expression at 24 hours compared to sham animals. After 72 hours, AQP4 expression increased in the pericontusional area as well as near lateral ventricles, but did not increase adjacent to the third ventricle. The hypothesis that the region-specific AQP4 expression in the brain after injury serves as a mechanism for fluid drainage remains a possibility. To understand the genesis of region-specific expression of AQP4 after TBI, the spatial distribution of molecules known to affect AQP4 such as thrombin, MMP, cytokines, and the activation of various pathways including p38, ERK, PKC, also need to be quantified in a spatial fashion.

Post-translational modification mechanisms of aquaporin-4

**Trafficking and membrane targeting of the AQP4 protein.**

The specific trafficking and endfeet-targeting mechanisms of AQP4 in astrocytes have not been explored in detail, but many studies have explored the general mechanisms of AQP4 assembly and targeting. AQP4 has six membrane-spanning domains. Immediately after the emergence of the polypeptide from the ribosomes, the translocon Sec61α inserts the six membrane domains sequentially into the ER membrane. After the translation event, the transition through Golgi requires the phosphorylation of AQP4 by protein kinase CK2 at several amino acid residues in the C-terminal domain. AQP4 is constitutively phosphorylated, and a quadruple substitution mutant of M1 AQP4 at Ser276, Ser285, Thr289, Ser316 which prevents its phosphorylation by CKII accumulates inside the Golgi apparatus. This evidence strongly suggests that the phosphorylation of these residues is required for the Golgi transition of AQP4.

AQP4 channels expressed on the membrane controls cell water permeability. While transcriptional activation of the aqp4 gene increases the amount of AQP4 transcript and protein, a more transient and rapid alteration in water permeability can be accomplished by the phosphorylation of AQP4 or by the internalization of AQP4. AQP4 is endocytosed through clathrin-mediated mechanisms via interactions of the tyrosine motif at the C-terminus with the μ subunit of the adaptor protein-2 (AP2) complex. AQP4 endocytosis is reduced by 80% when the Tyr277 was replaced with an Alanine, demonstrating the importance of the Tyrosine motif in the regulation of AQP4 endocytosis. A majority of the endocytosed AQP4 was directed to the lysosomes for degradation through the interaction with the μ subunit of AP3, and the lysosomal targeting of AQP4 is increased when Ser276 is phosphorylated by CKII. On the other hand, reversible AQP4 internalization can be triggered by histamine exposure, mediated through AQP4 phosphorylation by the PKA pathway. This pathway is colored in purple, in the lower portion of the signaling network.

**Phosphorylation and water permeability of AQP4 channels**

The phosphorylation of a protein at different amino acid residues can not only control the trafficking of the protein but also alter its function. Water permeability of AQP4 channels is increased through the phosphorylation of Ser111 by the activation of group I glutamate receptors (mGluR-I). The activation of mGluR-I can be induced by endogenous glutamate or an agonist DHPG. mGluR-I stimulation causes the release of Ca2+ from intracellular stores. The release of Ca2+ activates the production of NO, and PKG is subsequently activated. The increase of AQP4 water permeability by glutamate, an important neurotransmitter for the crosstalk between neurons and astrocytes, could be related to the need for maintaining homeostasis at the synapse and the water uptake driven by astrocytes after neuronal firing event.

On the other hand, direct phosphorylation of residue Ser180 will cause the inhibition of AQP4 by way of internalization. The AQP4 remains intact inside its cytosolic compartments after PKC-mediated internalization.
Therapeutic strategies to control expression levels of aquaporin-4.

AQP4 expression level is a deciding factor in the outcomes of cytotoxic edema, vasogenic edema, ischemia, hydrocephalus, and traumatic brain injury. Many efforts have been directed towards the development of pharmacological intervention therapies to improve disease outcome by targeting AQP4. While some disorders require the upregulation of AQP4, the downregulation of AQP4 is beneficial in others. However, the complex changes in brain microenvironment after most disorders implies that AQP4 has a dynamically changing role (either beneficial or deleterious) post-injury, and that the therapeutic regulation of AQP4 does not only require a simple trend.

Many molecular routes could effectively upregulate the transcription of AQP4, see Figure 75. However, the deleterious side effects of activating certain pathways renders most strategies not therapeutically desirable. This screening of therapeutically tractable molecular strategies targeting AQP4 requires a global understanding of molecular pathways.

Transcriptional upregulation.

Sulforaphane (SUL) has been found to upregulate AQP4 through Nrf2,(233) which also provides neuroprotective effects through the activation of genes containing anti-oxidant response element (ARE) region(234). SUL activates a neuroprotective transcription factor, Nrf2. Inactivated Nrf2 is bound to its inhibitory molecule Keap1 and this actin-bound complex is located within the cytoplasm. SUL is a lipophilic molecule that could travel across cell membrane. After entering the cytoplasm, SUL interacts with the Cys151 residue on the inhibitory molecule Keap1. It is commonly believed that Keap1 modification alone is not sufficient to cause the dissociation and translocation of Nrf2, and another residue on Nrf2, Ser40, needs to be
phosphorylated for dissociation and nuclear translocation to occur (235). PKC has been observed to play a role in the phosphorylation of Ser40 on Nrf2. Once inside the nucleus, Nrf2 binds to transcription factor MAF before binding to the aqp4 gene for transcription.

Other upregulators of AQP4 include tBHQ,(89, 236-237) vasopressin,(238) lactic acid,(239) mannitol,(141) testosterone,(240) IL-1β,(106) TNF-α,(195) and ammonia.(142) On the other hand, protein kinase C (PKC) activators including phorbol myristate acetate,(241) thrombin,(242) and tetradecanoyl phorbol acetate,(243) are known to cause AQP4 downregulation.

After controlled cortical impact injury, Guo et al. investigated the pericontusional areas, the lateral ventricles, and the third ventricle, and found no significant changes in AQP4 expression compared to sham animals or animals treated with progesterone. However, after 72 hours, AQP4 expression increased in the pericontusional area as well as lateral ventricle, but did not increase adjacent to the thrid ventricle. The upregulation of AQP4 after 72 hours was prevented and restored at almost normal levels after the administration of the neurosteroid progesterone. While this study confirmed progesterone administration was effective in reducing brain edema after TBI, the study did not unequivocally prove AQP4 expression as the cause for this improvement. Furthermore, this study demonstrated that expression changes of AQP4 were region specific. There is speculation about the observed regional difference in AQP4 as a mechanism to provide drainage for fluid, thus reducing cerebral edema formation regulating water balance, remains a possibility.

Transcriptional Inhibition of the aqp4 gene

The activation of PKC signaling pathways by PMA, TPA,(212-213) thrombin(211) and propofol(214) causes downregulation of AQP4, and this downregulation is caused by the decrease of mRNA at the transcriptional level.(212) Thrombin is released into the CSF after hemorrhage,(209) and the intraparenchymal injection of thrombin into rat brains induces BBB disruption which could be a possible mechanism of edema formation.(154) When analyzing the expression dynamics of AQP4 in injured brain, the effect of thrombin has to be taken into account. The downregulation of AQP4 by thrombin via the PKC pathway is partially mediated by PAR-1 receptor. PKC activation downregulates AQP4 in a time-dependent manner and PKC depletion caused by prolonged treatment of the PKC activator reverses AQP4 downregulation.(211)

MicroRNA-based therapeutics

Post-transcriptional control by miRNA is a way to induce changes in mature AQP4 channels. The injection of anti-miRNA320a in ischemic rats caused a 3.5 fold increase in AQP4 accompanied by a reduction in infarct volume.(216) In ischemic rat brains induced by middle cerebral artery occlusion, temporal plots of expression levels of AQP4 versus miRNA130a exhibit opposite trends, thus supporting the potential role of miRNA130a in regulating AQP4 expression levels. In addition, the injection of anti-miRNA130a to ischemic rat brains reduces the amount of miRNA130a to 37% and this reduction in miRNA upregulates AQP4 expression by 4 fold.(217) Moreover, miRNA130a specifically inhibit the M1 isoform of AQP4, but not M23 isoform, its specificity affords selective regulation of AQP4 isoforms. Translational regulation of AQP4 by miRNA can lead to differential expression of AQP4 isoforms during disorders like ischemia.

The translational inhibition of AQP4 transcript by miRNA is drawn in violet in Figure 73. The binding of the miRNA with Argonate family proteins is necessary for their subsequent localization in P-bodies. In P-bodies, miRNAs interact directly with decapping enzymes Dcp1 and Dcp2. Despite the promising use of miRNA as treatment therapeutics, each miRNA is predicted to have hundreds of down-stream targets. Therefore, microarray experiments are necessary to assess changes due to miRNA interference in the entire genome prior to the development of miRNA based therapeutics.
Conclusion.

AQP4 plays a pivotal role in neurological disorders, and it also contributes to the formation or the resolution of secondary brain injuries. Dynamic expressions of AQP4 after injury shows a complex pattern in the spatial dimension as well as in time. The design of AQP4 therapeutics needs to consider that AQP4 channels play different roles (beneficial or detrimental) in different injury phases after brain trauma. For example, after ischemic injury, AQP4 channels act detrimentally in cytotoxic edema, while they play a beneficial role in the resolution of vasogenic edema. To reach a desired pattern of AQP4 expression spatiotemporally means that the design of AQP4 molecular therapy needs meet to stringent design criteria.
Appendix 7. Modeling the dynamic response of aquaporin-4 channels for the design of molecular therapies - integration of kinetic models with subject-specific models.

Abstract

We propose to build an informatics platform for designing molecular therapies for tomorrow's medicine. This chapter describes a replicable and adaptable disease modeling methodology capable of integrating the abundant knowledge about cellular signaling, gene regulation and drug delivery models in systems biology with patient-specific medical imaging.

Many disorders of the central nervous system are tightly linked to the abnormal expression levels of a target gene. The over- or under-expression of the gene is often caused by altered intracellular signaling pathways. Many studies have investigated intracellular signaling and regulatory events in a single cell in normal and pathological states with computational models.(244-251) Furthermore, some studies have investigated the response of the target gene expression level after the application of a treatment.(252-253) These models quantitatively describe the response of a cell to extracellular stimuli and the induced changes in gene expression levels. By studying these signaling events, we can understand how abnormal gene expression levels are activated and how we can restore the cell to a normal state by pharmacological interventions. These cellular models that describe signaling, transcriptional and translational regulation events serve as road maps for designing molecular infusion therapies. However, there is a wide gap between quantifying the relationship between stress and induced gene expression in vitro and designing an infusion therapy which will cause a desired change in gene expression in an organism. We propose the coupling of the microscopic model describing cellular events with a model of the entire brain reconstructed from magnetic resonance images (MRI) of a patient to build an informatics platform with medical imaging information incorporated. We demonstrate for the first time the potential of organ-wide prediction of gene expression and protein levels in normal and disease states. These organ-wide models accelerate our understanding of the molecular perspectives of disease progression and serve as an in silico platform for testing pharmacological infusion therapies. We demonstrate in the case study the construction of a cellular model describing translational regulation of the aquaporin-4 gene. This model predicts how the levels of aquaporin-4 protein and mRNA change in response to an extracellular osmotic stress. A simplified version of this cellular model will be incorporated within a brain geometry to demonstrate the organ-wide prediction of gene expression levels. This adaptable and replicable platform predicts the concentration of disease-related proteins in tissue based on cellular signaling events, transcriptional and translational regulation. The computation of drug-organ interaction within a framework utilizing both physiological and biochemical information allows the determination of optimal dosage to successfully reach therapeutic endpoints. The integration of systems engineering, systems biology and medical diagnostic imaging will enhance our understanding of molecular perspectives of disease progression and accelerate the design of molecular therapies for tomorrow's medicine.
Introduction

Numerous disorders of the central nervous system (CNS) are tightly related to abnormal gene expression levels. Abnormal gene expression levels are often caused by altered intracellular signaling cascades that are induced by extracellular stress (254-256) or intercellular cross-talk (257-261). Many pharmacological agents are currently being developed to control the transcription and translation of a target gene. These molecular therapies have great potential in treating disorders such as cancer and chronic pain by restoring normal gene expression in affected cells.

For the development of molecular therapies, many genes involved in pathogenesis have been studied using cell cultures. In these studies, the relationship between an external stimulus (such as signaling molecules or neurotoxic chemicals) and the resultant changes in transcript and protein levels in cells is quantified. In some instances, the possible signaling mechanisms are postulated based on measurements of key signaling molecules. For example, the anti-angiogenic signaling mechanisms in vascular endothelium in response to endostatin treatment has been proposed based on in vitro observations (262). The endostatin-induced anti-angiogenic signaling has potential for the clinical treatment of cancer to reduce unwanted vascularization. On the other hand, the molecular circuitry of cancer centering around the tumor suppressor protein p53 has been established (263). The targeting of specific cell type and tumorous regions in an organ demands patient-specific information about anatomy and physiology. To successfully implement these treatments in vivo requires not only an understanding about gene expression but also an effective drug delivery system. The generation of organ-wide gene expression maps based on systems biology knowledge requires an integrated platform between medical diagnostic imaging and cellular biochemistry. This platform will predict patient-specific gene expression changes in normal and disease states. In addition, this integrative approach will predict the dynamic changes in signaling, transcript levels and local protein concentrations in response to the injection of a potentially therapeutic molecule. In this chapter, we demonstrate the construction of such an informatics platform and its applications in designing novel molecular therapies.

Despite a rich knowledge base in cellular signaling networks, the organ-wide prediction of gene expression, signaling events and induced changes in protein levels is still in the budding stage. The outcomes of molecular therapies will be difficult to predict basing solely on experimental observations in vitro, without considering the complexity in anatomy, the heterogeneity in cell types, the effectiveness in drug biotransport and the dynamics of drug-cell interaction on an organ-wide level. Despite the advance of high resolution medical imaging techniques, the integration of the macroscopic anatomy, patient physiology, and the microscopic cellular biochemistry has not been thoroughly investigated. There is a need to integrate clinically relevant anatomical and physiological information with systems biology models for making organ-wide predictions of gene expression. Whether or not an infusion therapy will cause the desired change in protein levels is functions of the accessibility of the target tissue, drug biodistribution and metabolism, cellular uptake, as well as subsequent signaling and transcriptional events.

We can design more effective therapies by integrating systems biology models with patient-specific medical imaging technologies.

A case study about AQP4 distribution in the brain based on translational dynamics will be used to demonstrate this platform. AQP4 is a water channel found predominantly in the central nervous system (CNS) (75). This water channel has an important role in regulating water transport, cell volume and ionic environment inside the CNS (264-265) and it is implicated in life-threatening disorders such as hydrocephalus (84) and edema (139, 266-268). While hydrocephalus is caused by excess accumulation of cerebrospinal fluid (CSF) inside the ventricles, brain edema usually entails fluid accumulation inside the cells or within the extracellular space of the CNS. Understanding the transcriptional and translational regulatory mechanisms of AQP4 will accelerate the development of pharmacological interventions to up- or down-regulate AQP4. Controlling with pharmacological agents the transcriptional activation or translational regulation of aquaporin 4 gene (aqp4) can potentially restore water balance. In the first case study, we will integrate a proposed translational regulatory mechanism of AQP4 with an in silico brain model. This case study shows how a systems biology model describing translational regulatory mechanism of a target protein can be integrated with medical imaging to compute the organ-wide distribution of this protein in the entire brain.
Construction of an anatomically consistent model of a target organ

For organ-wide gene expression analysis, the reconstruction of the organ anatomical geometry is a crucial first step. For gene expression analysis in the brain and the spinal cord in the following case studies, the exact anatomy of the CNS is reconstructed from MRI of a subject using a procedure described elsewhere. (36, 71, 269-271) The anatomical geometry of the CNS in the reconstructed models has subject-specific geometry, and this accurate reconstruction of the anatomy is important in the analysis of drug distribution patterns and the prediction of therapeutic effect for a specific subject after an infusion therapy.

A patient-specific model was reconstructed from MRIs of the patient's CNS through a process termed image reconstruction (Figure 76). (36, 71, 269-271) In an image reconstruction software - MIMICS innovation suite, the anatomical geometry of the brain tissue, spinal cord tissue, and the surrounding CSF were captured by automatic and manual segmentation. The brain was divided into the grey matter, white matter and different functional regions such as the pons. Different drug transport properties, extracellular space fraction, cellular composition, anisotropic diffusion and endocytosis rates can be assigned to each region, reflecting physiological complexity of the brain. (272) This subject-specific model was converted into an unstructured computational mesh as described by Somayaji (71) for the organ-wide analysis of gene expression, protein levels, and predicted outcomes of molecular therapies.
Aquaporin-4 (AQP4) is a water channel in the brain that is mainly expressed in cells around the BBB or tissue-CSF boundaries. The conduction of water by AQP4 channels is induced by an osmotic gradient. The effective upregulation of AQP4 in the brain aids in the restoration of water balance during vasogenic edema in animal models. However, the design of new therapies for the upregulation of AQP4 presents two main challenges. First, due to the complex architecture of the brain, the concentration of drugs reaching a target region depends on the delivery mode, infusion parameters, drug molecular properties, brain tissue anisotropy and heterogeneity, and anatomical brain geometry of a particular patient. Second, it is difficult to quantify the expected change in AQP4 transcript and protein levels after an infusion of a molecular agent. In targeted cells, the degree of AQP4 upregulation depends on how the drug interferes with the transcriptional and translational regulatory mechanism. This translational approach uses state-of-the-art medical imaging to generate a patient-specific model of the brain, and this macroscopic organ model is integrated with microscopic cellular model that describes translational regulation events. This integrated model can be used for testing specific molecular strategies to enhance transcription or translation based on aqp4 gene regulation and signaling.

**Case study: translational regulation of aquaporin-4 in the entire brain**

Aquaporin-4 (AQP4) is a water channel in the brain that is mainly expressed in cells around the BBB or tissue-CSF boundaries. The conduction of water by AQP4 channels is induced by an osmotic gradient. The effective upregulation of AQP4 in the brain aids in the restoration of water balance during vasogenic edema in animal models. However, the design of new therapies for the upregulation of AQP4 presents two main challenges. First, due to the complex architecture of the brain, the concentration of drugs reaching a target region depends on the delivery mode, infusion parameters, drug molecular properties, brain tissue anisotropy and heterogeneity, and anatomical brain geometry of a particular patient. Second, it is difficult to quantify the expected change in AQP4 transcript and protein levels after an infusion of a molecular agent. In targeted cells, the degree of AQP4 upregulation depends on how the drug interferes with the transcriptional and translational regulatory mechanism. This translational approach uses state-of-the-art medical imaging to generate a patient-specific model of the brain, and this macroscopic organ model is integrated with microscopic cellular model that describes translational regulation events. This integrated model can be used for testing specific molecular strategies to enhance transcription or translation based on aqp4 gene regulation and signaling.

**Hydrocephalus and expression levels of aquaporin-4.**

Studies have shown that kaolin-induced hydrocephalic weanling rats show an initial decrease in AQP4 expression in both the periventricular and cortical regions of the brain. The AQP4 level returns to normal level after one week, and rises significantly above normal afterwards. This dynamic trajectory of AQP4 still cannot be explained. In many CNS disorders, the osmotic environment changes in the brain due to the secretion of inflammatory cytokines, accumulation of ions, and extravasation of proteins from the cerebral vasculature. We propose a possible mechanism of translational regulation of AQP4 transcript that could be one of the contributing factors for the transient decrease in AQP4 water channels in the initial stages of hydrocephalus.

**Proposed translational regulatory mechanism of aquaporin-4 in a single cell**

When an osmotic stress is present, the cells exhibit an "osmoprotective response" to repair DNA damage and restore cell volume. During this stage, the translation of the majority of mRNAs is halted, while the translation of some selected mRNAs is accelerated. The majority of the mRNAs enter storage temporarily until released to rejoin the translation process. A transient decrease in protein levels could be related to the temporary translational inhibition of these mRNAs. Based on the dynamics of mRNA translation and storage, we propose a translational regulatory mechanism of AQP4 mRNA.

In yeast, the hog1 signaling pathway is analogous to the p38 mitogen-activated protein kinase (MAPK) pathway. The hog1 pathway controls the production of yeast aquaporin (AQY), the equivalent water channel found in yeast cells. To study the response of the transcriptome to osmotic stress in yeast, the transcript and protein levels after exposure to osmotic stress were quantified in a study by Melamed. The portion of actively translating AQY mRNAs found in polysomes (association of multiple ribosomes) is compared to the portion of those AQY mRNAs that were not being translated efficiently (with only one ribosome attached or without any ribosome). After the induction of osmotic stress, the maximum inhibition of translation occurred after approximately one hour. The portion of the mRNAs in the polysomal pool drops from 75% at normal conditions to 10% under osmotic stress conditions. The translational response to osmotic stress precedes the transcriptional response. The ratio of translational efficiency of AQY mRNA in normal and under osmotic stress conditions was evaluated by fractional comparison, see equation (29).

A number greater than 1 indicates the increased translational efficiency of that mRNA during osmotic stress. A number between 0 and 1 indicates the inhibition of translation for that mRNA during
osmotic stress. The ratio of 0.146 indicates that the mRNA of AQY is translated about 8 times less efficiently during osmotic stress compared to during normal conditions. (275)

\[
\frac{\text{polysome}_{\text{osmotic stress}}}{\text{polysome}_{\text{normal conditions}}} = 0.146
\]  

(29)

We propose a translational regulatory mechanism for AQP4 in mammalian cells based on the observations in yeast AQY. Even though there are likely differences in the translational regulatory pathways of mammalian AQP4 and yeast AQY, some homology is expected. The fate of AQP4 mRNA is described below. After exiting the nucleus, the mRNA is bound to ribosomes, creating a polysome. (276) The ratio of polysomes versus free mRNAs indicates the translation efficiency. The decapping promoter and enzyme complex (decapping complex) binds to the 5’ end of the polysomal mRNA, (276) removes the poly(A) tail (277-278) and recruits the degradation protein complex (Deg) to begin 5’ to 3’ degradation (279-280) as the translational ribosomes fall off. However, during osmotic stress, (281) the poly(A) tail is instead decapped by the decapping complex. (277-278) This complex recruits the binding proteins for the stabilization and storage of AQP4 mRNAs during osmotic stress. These binding proteins bind to the 5’ end of the mRNA strand, and prevent the degradation enzymes from degrading the mRNA. The decapped mRNA-binding protein complex will aggregate into processing bodies (P bodies) in the cytoplasm around the nucleus. (277-278, 280, 282-284) The stored mRNA can enter stress granules, (284-285) which contains recapping factors like poly(A) protein, poly(A) tail, and translation initiation units (40s). (278, 285) The species involved in the proposed translational regulatory mechanisms are described in Table 17.

Table 17. Species for aquaporin-4 translational mechanism

<table>
<thead>
<tr>
<th>Species</th>
<th>Species Description</th>
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<tbody>
<tr>
<td>Decapped mRNA</td>
<td>Decapped mRNA</td>
</tr>
<tr>
<td>Stored mRNA</td>
<td>Stored mRNA</td>
</tr>
<tr>
<td>Stress Granules</td>
<td>Stress Granule, consisting of Poly(A) protein and 40s ribosomal proteins</td>
</tr>
<tr>
<td>Stress Granules + polyA</td>
<td>Stress Granule with Poly(A) tail</td>
</tr>
<tr>
<td>Decapping</td>
<td>Decapping promoter (Dhh1p) and enzymes complex which can recruit either Deg or Binding proteins</td>
</tr>
<tr>
<td>polyA</td>
<td>Poly(A) tail, is removed via Dhh1pdec</td>
</tr>
<tr>
<td>polyA protein</td>
<td>Poly(A) tail binding protein, re-attaches Poly(A) tail to decapped mRNA</td>
</tr>
<tr>
<td>40s</td>
<td>Translation initiation ribosome, usually resides in Stress granules</td>
</tr>
<tr>
<td>Deg</td>
<td>Degradation proteins, degrade the decapped mRNA from 5’ to 3’</td>
</tr>
<tr>
<td>Binding proteins</td>
<td>Binding proteins, prevent Deg from binding and degrading mRNA</td>
</tr>
<tr>
<td>Polysome</td>
<td>mRNA bound to ribosomes, a translating mRNA complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>AQP4 mRNA</td>
</tr>
<tr>
<td>Rib</td>
<td>Ribosomes for translation</td>
</tr>
<tr>
<td>AQP4</td>
<td>AQP4 protein</td>
</tr>
</tbody>
</table>
Based on the proposed mechanism, the dynamic changes in AQP4 transcript and protein levels in mammalian cell is predicted using a system of ordinary differential equations and a stochastic algorithm termed Gillespie algorithm. Kinetic rates are assigned, and some kinetic rates vary as functions of the osmotic stress to reflect dynamic changes in biochemical reaction rates during the exposure of the cell to osmotic stress. Figure 77 shows the dynamics evolution of key intracellular species in this proposed translational regulatory system. In Figure 77, the translation process of AQP4 progressed at a steady rate for the first 1000 seconds (0.278 hour). When an osmotic stress is introduced at 0.278 hour, the translational dynamics begin to change. First, we observe a rapid accumulation of stored AQP4 mRNA. The number of AQP4 mRNA in actively translating polysomes decreased from 47 per cell to below 5 per cell, indicating a halt in the translation process, in agreement with observations in yeast cells during osmotic stress. As a result of this translational inhibition, a decreased expression of AQP4 channels is observed.

Organ-wide expression of aquaporin-4 water channels

![Figure 77. Translational dynamics of aquaporin 4 mRNA and key intracellular molecules during steady state and osmotic stress. The proposed translational regulatory mechanism was formulated deterministically with ordinary differential equations (smooth curves) as well as stochastic Gillespie algorithm (jagged lines).](image)

A simplified version of the single-cell model described above was integrated with a brain model to generate an organ-wide mapping of AQP4 transcript and protein levels. Based on the observation that AQP4 expression levels are higher near brain-CSF boundaries, some kinetic rates are made functions of the distance from the location of the cell to nearest brain-CSF interface.

The predicted levels of AQP4 channels as well as mRNAs are shown in Figure 78. The computational results shown high density of AQP4 around brain-CSF boundaries and periventricular regions as seen in the autoradiography image in the left panel of Figure 78. The copy numbers of mRNA molecules per cell are predicted. The generation of brain-wide gene expression map of AQP4 will accelerate the design of molecular therapies.
Controlling the expression of disease-related genes in the CNS using molecular therapies is the next generation of CNS treatments. However, CNS therapy design poses stringent requirements. The traditional trial and error animal infusion experiments do not provide quantitative answers for the optimal dosing of a human subject. This article addresses the challenge of optimal human dosing by integrating medical imaging with systems engineering principles. The prediction of brain-wide expression pattern of AQP4 is only the first step. The integration of fluid dynamics to describe drug biotransport after an intrathecal infusion is the logical next step. However, this platform brings the study of gene regulation, protein expression, and the quantitative relationship between a molecular drug and induced cellular response to an organ wide level. Future direction includes the coupling of this patient-specific model with spatially distributed kinetic inversion technique for the determination of unknown reaction parameters using medical imaging.

Conclusion

The expression level of a gene is controlled by complex intracellular signaling networks, transcriptional and translational regulation mechanisms. Many therapeutic compounds are being developed to alter gene expression. Numerous mathematical models in the field of systems biology have quantified cellular signaling and gene regulation events in normal, disease, and treatment phases. Many of these works point to the promise of molecular therapies that will control and reverse the state of pathological gene expression in a targeted organ. However, a translational platform for integrating the systems biology knowledge base with medical imaging does not exist. How microscopic gene regulatory events could be incorporated to better molecular therapy design is still unclear.

We have developed an adaptable and powerful informatics platform that combines anatomical information from medical imaging with microscopic events about gene regulation and control. With this platform, single-cell predictions of dynamic changes in protein and transcript levels in response to disease progression and molecular treatment can be transformed into an organ-wide response prediction. The integration of drug biotransport, tissue properties, fluid dynamics, and cellular biochemistry will enable researchers to conduct drug infusion experiments in silico and monitor the organ-wide response. Although this model will not replace the value of infusion tests, it can reduce cost of trial and error experimentation by generating reasonable predictions of dose-response. The prior prediction of dose-response, range of optimal infusion parameters, and even toxicity maps in surrounding tissues can drastically accelerate the design of novel therapies. In addition, computations of drug action, bioaccumulation, and induced changes in gene
expression enable the prediction of therapeutic efficacy a priori. This novel approach at the interface of systems biology, medicine and engineering brings us closer to personalized infusion therapy design for tomorrow's medicine.
VITA
Ying Hsu

Education
M.S. in Bioengineering, University of Illinois at Chicago (Thesis Advisor: Andreas Linninger)
B.S. in Bioengineering, University of Illinois at Chicago (Honors College) - Graduation: May 2010

Honors and Awards
Best Poster in Category at 2012 Annual Conference of International Anesthesia Research Society (IARS)
First Place Kosaka Award for Best Presentation at 2012 Annual Conference of IARS
2nd Place 2012 MIMICS Innovation Award Winner
2nd Place (co-author) in 2012 conference in Science, Technology, Engineering, and Mathematics (STEM)
Society of Engineering Science 2011 Conference travel award and invited speaker
Society of Engineering Science 2010 undergraduate presentation competition Finalist
Society of Engineering Science 2010 Conference NSF travel award
Honors College Member, University of Illinois at Chicago
Honors College Tuition Waiver Recipient (four terms)
Chancellor’s Student Service Award (Spring & Fall 2006)
Inder P. and Uma Batra Memorial Undergraduate Physics Award for 1st class performance in physics

Work Experience
Jan 2011- present Graduate Research Assistant
- build patient-specific computational models through medical image reconstruction
- drug infusion simulations, drug pharmacokinetics and toxicity risk analysis
- supervise undergraduate researchers
- organize meetings with medical collaborators
Sep 2008-June 2009 Office translator, University of Seville, Spain (student exchange work-study)
- translate documents from Spanish to English
May 2005- Jan 2007 Starbucks Coffee Company
- prepare high quality coffee beverages
- represent the brand and provide customer service focused on effective communication

Publications


**Conference Presentations**

May 30th - June 1st, 2012  **RNAi Therapeutics**, Boston, MA. **Invited Lecture**: Improving gene silencing efficacy in vivo with organ-wide quantitative design of siRNA infusions.


Nov 12th, 2010  **College of Medicine Research Forum**, University of Illinois at Chicago, IL. **Poster presentation**: Intrathecal Drug Delivery to the Human Central Nervous System.
