Cryopreservation of cells and tissues offers a host of benefits to a broad spectrum of fundamental sciences and can be modeled as a series of heat and mass transport systems, which can be employed to formulate well defined optimal control problems governed by ordinary and partial differential equations. The governing transport models and their controls encompass multiple scales and domains providing a rich resource for mathematical research. Amazingly, there are few other mathematicians working directly in this exciting and fruitful area. Over the last 15 years, I have been in one way or another involved in this field, with activities ranging from developing numerical and analytical optimal control methods to microsurgery on hamsters. I have worked with many biologists, engineers, physicists, and mathematicians on a huge variety of problems, and have gained real world applied mathematical experiences along the way. For this interdisciplinary work, I was awarded the University of Missouri's highest graduate student research honor, the Donald K. Anderson Graduate Research Award, in 2007. In 2008 I was awarded the Peter J. Steponkus Crystal award for best student presentation at the annual Society for Cryobiology meeting, and in 2009 I was asked to serve on the editorial board for the journal *CryoLetters*. Finally, because of contributions in the field of Cryobiology, I have been nominated by the Society for Cryobiology board of governors to the electoral slate of board of governor candidates in both 2009 and 2010. These experiences give me a unique skill set that will sustain a long and productive career in applied mathematical research.

From a global perspective, cryobiology has huge financial, ethical, agricultural and scientific impacts and because of this widespread need for optimized cryopreservation, there have classically been multiple sources of funding available to researchers in this interdisciplinary field, including the NSF, NIH, USDA, DARPA, NASA, and industrial and private funding sources. I have had direct experience writing and co-writing several NSF and NIH grants, two of which were funded during my studies at the University of Missouri. Additionally, my National Research Council postdoctoral research proposal sparked enough interest to garner an NRC-NIST postdoctoral associateship, under the direction of Anthony Kearsley.

In this document, I outline my mathematical contributions to an array of problems, most requiring new methods and new approaches—purely biological contributions are outlined in my CV. From this document you will find that there is no shortage of interesting problems in the field of mathematical cryobiology; that I am uniquely qualified to contribute in new ways to this field; that I have learned from each new project and expanded my mathematical horizons to make me an applied mathematician with a large tool-set; and that I have a specific research plan that has both mathematical depth along with potential for significant impacts on cryobiology.

Past research

Optimal control

Because it is dominated by heat and mass transport and has well defined costs and constraints, cryobiology is a perfect setting for the application of optimal control. To

my knowledge, however, only one other group, headed by K. H. Hoffmann in Germany is actively researching this area, making this field nearly wide-open.

Before (and after) cryopreservation, cells must be equilibrated with (and from) high concentrations of permeating chemicals called CPAs which mitigate the effects of freezing. This equilibration, however, produces two competing deleterious factors: potentially damaging osmotic volume fluxes and time-dependent chemical toxicity. These factors, then, define a classic state constrained time-optimal control problem governed by the n dimensional system of nonlinear ODEs:

$$\begin{aligned} &\text{find} \ \min_{M_i \in \mathcal{A}} \{t^f \in \mathbb{R}^+ : w(t^f) = w^d\} \quad \text{subject to} \\ &\left.\begin{aligned} \dot{w}_1 &= x_{np}/w_1 + \textstyle\sum_{i=2}^n w_i/w_1 - \sum_{i=1}^n M_i, \\ \dot{w}_i &= b_i(M_i - w_i/w_1) \quad i \in (2, \ldots, n), \end{aligned}\right\} \\ &k_* \leq \Gamma \cdot w \leq k^*. \end{aligned} \tag{1}$$

My first result was to note that by the nonlinear time transformation,

$$q(t) = \int_0^t x_1(\tau)\, d\tau, \tag{2}$$

this 90-year-old nonlinear ODE has analytical solutions [6]. Because of this, analytical calculations of important cryobiological endpoints could be made. Furthermore, with this transform as a tool, we convert this nonlinear problem into a linear one, facilitating a proof of stability, controllability, and existence results in the case of an arbitrary number of permeating solutes [8].

We synthesized optimal controls for the more tractable and most used two dimensional state space: given any initial and final condition we prove that this synthesis was "regular and distinguished," necessary and sufficienct optimality conditions using the geometric optimal control theory result of Boltayanskii [8, 11]. Because of this complete synthesis, we were then able to overlay the natural linear inequality constraints that are generated by the maximal and minimal volumes or concentrations tolerable by the cells [9].

This general result yields specific insight into cryoprotocols with far-reaching implications for cryobiology. For example, red blood cells are frozen in media containing 40% glycerol that must be removed from the blood before clinical use. Using current methodology this process takes around 45 minutes. Using optimal control theory, we predict that the theoretical minimum dilution time can be reduced to less than 7 minutes. The implications of this optimization is that 6 times as much blood could be made available to patients. This process can be extended to other important and sensitive cell types such as human oocytes, mouse and rat sperm, and embryonic stem cells with similar 5-7 fold reductions in exposure time.

Mass transport

The penalty of this optimal control is that it requires continuous control of the media concentration around the cells. The most natural way to achieve this is to continually

perfuse media over cells held in place by a membrane. We hypothesized that this perfusion might introduce significant advective effects and constructed the axially symmetric model, with $\Omega_t = \Omega \setminus \Omega_1$,

$$\left.\begin{aligned} c_t &= D\Delta c + u \cdot \nabla c \quad (x,t) \in \Omega_t \times [0,\infty), \\ c &= c^e \quad (x,t) \in \partial\Omega_t \times [0,\infty), \\ dc/d\eta &= \alpha(c - c^c) \quad (x,t) \in \partial\Omega_1[0,\infty), \end{aligned}\right\} \tag{3}$$

$$\left.\begin{aligned} \Delta\Psi &= 0 \quad (x,t) \in \Omega_t \times [0,\infty), \\ \Psi &= \Psi^e \quad (x,t) \in \partial\Omega_t \times [0,\infty), \\ u &= \nabla\Psi, \end{aligned}\right\} \tag{4}$$

where $c^c = \mathcal{M}c|_\partial\Omega_1$, $\mathcal{M}$ a solution operator for equation (1), and $\Omega_1 = \Omega_1(w)$ is not fixed. We define a time-varying state transformation so that this free-boundary problem was converted to a fixed boundary problem faciltating exact solutions of system (4), leaving the two solute quasi-linear system (3). We developed a numerical scheme to determine the relationship between flow rate and concentration distribution along the cell/tissue boundary. Corresponding to Péclet number arguments, at single-cell scales, diffusion dominates at all practical flow rates, but for reproductive cell and small tissue scales and larger, significant concentration gradients along the cell or tissue boundary build up at practical flow rates, and solute polarization at low flow rates has a significant effect on the concentration gradients and thus theoretically optimized procedures [7]. I presented this work at the Society for Cryobiology 2008 Annual Meeting in Charlotte, North Carolina, and was awarded the society's highest student honor, the Peter J. Steponkus Crystal Award.

Parameter estimation

Research performed under a grant written with John Critser and postdoc Xu Han included developing two independent methods to measure the permeation of ethylene glycol into mouse ovarian tissue. Noisy data required the homogenization of the radially symmetric linear diffusion equation to yield a first-order approximation of CPA diffusivity. These novel data provide insight into the development of optimized cryopreservation protocols, and in our manuscripts we prescribe a dramatically new heuristically optimized CPA loading protocol [14, 15].

Optimization of human Endothelial Progenitor Cell (hEPC) and mouse Embryonic Stem Cells (mESC) cryopreservation

In collaboration with Dr. Erik Woods of Indiana University, I measured hEPC volumetric response to anisosmotic conditions using a Coulter counter. Data from this device is particularly noisy, so I created a maximum liklihood scheme that took advantage of the form of the exact solution mentioned above to reduce the data and another nonlinear least squares minimizing scheme to estimate the temperature dependent rate parameters $b_i := b_i(T)$ of the ODE mass transport model (1). I then created an iterative numerical algorithm using these data to determine the optimal (constant) cooling rate (ρ). Specifically, I solved $\max 2 = R\rho$ subject to the constraint

that system (1) with temperature $T(t) = T_0 - \rho t$ was supercooled no more than 2 K–constant cooling rates were a limitation of the cooling devices. Supercooling was determined by coupling system (1) with a phase diagram and cooling protocol [10].

A postdoc in our lab, Corinna Kashuba Benson, undertook a similar study to optimize freezing protocols of mESC. She collected the volumetric data that I again reduced, fit to the transport model, and used to predict optimal freezing protocols. My optimization was applied and she has achieved a doubling in post-thaw recovery [2]. We have completed this study in multiple ES cell lines, and have improved recovery in all tested lines, with a manuscript in preparation.

Mathematical Chemistry

From the above work, it can be seen that optimized freezing protocols depend on the functional relationship between melting point and the concentration of the principle components of the media now well described by two theories of additive osmolalities. These theories must be validated in unknown systems, and we have done so in a ternary system with a differential scanning calorimeter (DSC). In collaboration with Erik Woods, John Critser and several others, I developed and fit the theoretical models to data for the ethylene glycol-sodium chloride-water, resulting in a manuscript [4].

Current and future research

Cost functions

Present I presented my state constrained minimal-time optimal control results at the 2009 annual meeting of the Society for Cryobiology. Afterwards, I was approached by several engineers and biologists who were interested in applying my theory. It seems that the impact of my work may be that experimentalists are now beginning to think in terms of minimizing cost functions instead of simply testing "optimized" protocols.

In particular, Adam Higgins (Oregon State University) presented work, based on my results, at the 2010 annual meeting of the Society for Cryobiology, showing that a more cost function was $J(M) = \int_0^{t^f} c^2(s)\, ds$, where t^f is some final time, and c is the intracellular concentration. He was anxious to work with me to apply this research in a more sophisticated mathematical setting, and, along with Tony Kearsley, we have used a direct optimization method to solve the constrained optimal control problem, defining unexpected and novel addition and removal protocols [16].

Future This collaboration has led to a clear research path to define temperature dependent costs so that an entire protocol may be optimized. In other words, we wish to first define and then minimize the cost functional $J(M, T)$ with time dependent temperature T and concentration M subject to the heat and mass transfer of the entire cryo-protocol. No researcher has used optimal control to account for both CPA equilibration and removal protocols and temperature profiles to minimize a single cost

functional. The objective determination of appropriate cost functions for cryopreservation promises to be fundamental to the field, as well as fruitful mathematically. This will lead to new constraints and new model formalisms, and will require refined approaches to optimization.

Decoupling of control domains

Present The mass transport models used in my current work with Higgins suppose perfectly stirred media. My thesis work addressed this issue by coupling an advection diffusion equation for mass transport with a simple fluid flow model and showed that there were significant effects of both advection and diffusion that are not usually accounted for in the standard models which leads to challenging state and PDE constrained control problems.

To simplify I observed that non-ODE control in cryobiology almost always is of the following form. Suppose that $\Omega_1 \subset \Omega$ are closed, connected subsets in $\mathbb{R}^3$. Typically, we may think of Ω_1 as the biological material and $\Omega \setminus \Omega_1$ as the surrounding materials (e.g. media, container, heat source, etc.). In cryobiology, we ultimately do not care what happens in $\Omega \setminus \Omega_1$, and thus the cost function for a control system where we control some quantity at the boundary of Ω ($\partial\Omega$) is almost universally evaluated only in $\Omega_1 \cup \partial\Omega$.

The enormous advantage of this is that one may decouple the optimal control problems. First optimize in Ω_1 to determine optimal controls v_1 on $\partial\Omega_1$. Then optimize in $\Omega \setminus \Omega_1$ to determine appropriate optimal controls that approximate v_1 at $\partial\Omega_1$. This amounts to solving an optimal control problem and an inverse problem. In the 1D setting with the linear diffusion equation governing mass transport, I have proved that one may solve this inverse problem analytically, and thus, coupled with the single cell analytical optimal controls, analytical optimal controls of the coupled, constrained, ODE-parabolic system may be derived. The advantage of this approach is that it gives a very rich test bed for the numerical optimization that must be used in higher dimensions with nonlinear heat transfer. In fact, I have performed numerical optimization using an hp-adaptive elliptic solver to solve the 1-D parabolic system (PHAML, [18]) coupled with a semi-implicit filtering algorithm [13] which uses a secant approximation of the gradient to minimize the cost functional. This numerical approach well approximates the analytical approach.

Future Using the tested combination of PHAML and optimization algorithm along with the decoupling technique, I intend to solve a series of optimal control problems. First, given measured parameters from previous experiments, determine optimal initial—pre-cooling—concentration distribution in axially symmetric tissues given an a priori known maximal cooling rate. Then, given the optimal initial concentration distribution determined above, and toxicity cost functional defined with Adam Higgins, determine optimal boundary ($\partial\Omega_1$) concentrations to achieve the optimal initial concentration in the previous step. Finally, with the mass transport model developed in my thesis, solve the inverse/boundary-control problem in $\Omega \setminus \Omega_1$ to determine optimal concentration and fluid velociies at $\partial\Omega$. This process can be

further constrained by mandating that minimal concentration gradients exist along $\partial\Omega_1$.

Mass transport systems

Present My involvement with the EG phase diagram sparked an interest in whether ODE mass transport based on this new chemical potential model had any intrinsically different behavior compared to the classical model I have used. Following the approaches developed by Elliott et al. [12], I derived an n-solute system of mass transport ODEs under several different modeling formalisms, showed that their behavior under linearization around a rest point was identical, and showed that instabilities should occur only under unphysical model conditions [3].

Mass transport modeling in cryobiology occurs on three length scales: cell, multicell, and large tissue, corresponding to 10^1 μm, 10^2 μm, and 10^3 μm, respectively. At the small and large scales, mass transport is fairly well understood, but there is little consensus for the middle scale. Charles Benson, John Critser and I developed a model to account for cell-cell, cell-interstitum, and interstitial-diffusive fluxes. The approach used a tortuous reaction-diffusion equation subjected to a domain-decomposition technique to account for the local interaction of cells and the surrounding envirionment. The nonlinear system was solved via the method of lines and the only phenomenological parameter fit for was the cell-interstitial surface area. The coefficient of determination was good indicating that this model captures a large portion of the geometric information inherent in the system [5].

I am currently working with Dan Anderson (George Mason University) to define a coupled heat and mass transport model that accounts for the approaching solidification front, coupled with a moving cell or tissue boundary. At the extreme viscosities and temperature ranges encountered during cooling in the presence of high concentrations of cryoprotectants, classically used level-set approaches are not as appropriate. Our approach follows that of Anderson's past work involving so-called mushy layers [1], where the state is at a transition between solid and liquid. We expect that there may be diffusion limited ice growth along with a complex interaction between biomaterial and its envirionment that is not currently accounted for in models of solidification in cryobiological settings.

Future I intend to use the numerical techniques developed in previous research to develop optimal protocols based on these models.

Iterative cryopreservation optimization

Future Mathew Tomlinson (University of Nottingham) and I have formed a collaboration to systematically optimize human sperm cryopreservation. My approach will be to apply a nonlinear programming approach to minimizing a cost function defined by quickly measurable post-procedure end points. This technique has been used for the optimization of mass spectrometry calibration [17] and is the best approach for optimizing sperm cryopreservation because—despite best efforts—optimal design

of sperm cryopreservation using classical models have failed, most likely due to the unique geometries and functions that sperm exhibit.

If this approach is successful, this may be a powerful compliment or even alternative to the biophysical approaches I have defined above. In particular, valuable and challenging cell types where this approach may work are human embryonic stem cells, where cells must be preserved in non-homogeneous clusters, and thus modeling approaches are more challenging, and large quantities of cells are available, facilitating a large number of permutations and faster convergence.

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