

# **Characterization of Primary Rat Astrocytes: a Step Towards Optic Nerve Head on a Chip**

A Thesis  
Presented to  
The Academic Faculty

by

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In Partial Fulfillment  
of the Requirements for the Research Option in the  
School of Biological Sciences

Georgia Institute of Technology

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## ABSTRACT

The mechanism by which elevated intraocular pressure (IOP) increases risk of glaucoma remains unknown. A proposed pathway suggests astrocytes sense optic nerve head (ONH) deformation due to increased IOP, triggering their conversion to a reactive phenotype<sup>1</sup>. When reactive, they can promote axonal damage in retinal ganglion cells (RGCs), causing progressive vision loss in glaucoma patients<sup>4</sup>. Most research aimed at understanding astrocyte mechanobiology has been conducted in monolayer culture systems using DiTNC1 cells, a brain-derived line of cells, rather than primary ONH astrocytes<sup>13</sup>. Such 2D cultures are problematic because they induce baseline activation, restricting our ability to understand true cellular response to stressors<sup>8,9</sup>. Based on research using 3D culture systems that yielded preliminary results with DiTNC1 cells, we aim to develop a 3D culture system that can be used to elucidate astrocyte mechanotransduction pathways using primary ONH astrocytes and contribute to the development of accurate *in vitro* models of glaucoma<sup>10,11</sup>.

Characterization of isolated rat ONH astrocytes revealed positive staining for the astrocyte markers connexin-43, glial fibrillary acidic protein (GFAP), and vimentin. These astrocytes can be used to more accurately model and understand glaucomatous pathology using our 3D culture system. With the hope of ultimately developing an “optic nerve head on a chip,” our model system will expedite assessment of potential astrocyte-targeted therapeutics, facilitating the identification of alternative and/or complementary treatments to current IOP lowering strategies.

## INTRODUCTION

Glaucoma is an optic neuropathy recognized as the principal cause of permanent vision loss worldwide. It affects approximately 64 million individuals, a number projected to double in the next twenty years<sup>2</sup>. All current therapies aim to lower IOP, the primary known risk factor for glaucoma<sup>1</sup>. These treatments typically involve a cumbersome regimen comprised of expensive medications and multiple eye-drop administrations throughout the day. Americans alone spend an estimated \$2.9 billion annually to manage their glaucoma<sup>3</sup>. Combined, these factors can often result in poor patient compliance. Additionally, present therapeutics mainly decelerate, not treat or reverse, vision loss<sup>1</sup>.

In order to formulate more successful treatment options, it is imperative that we better understand glaucomatous pathology. Researchers have proposed that optic nerve head deformation (ONH), instigated by elevated intraocular pressure (IOP), causes astrocytes to transition to a reactive phenotype<sup>5,6</sup>. Astrocytes are mechanosensitive glial support cells that maintain the matrix surrounding RGCs<sup>7</sup>. When reactive, their altered expression may induce axonal degradation in RGCs, prompting progressive vision loss<sup>4</sup>. However, the role of astrocyte activation and reactivity in glaucoma remains unknown. We propose that alteration of matrix properties leads to chronic astrocyte reactivity, which has been implicated in glaucoma, but we need a physiologically relevant system to be able to interrogate this suggested relationship.

We aim to create a high throughput 3D astrocyte culture system that will contribute to the development of accurate *in vitro* models of glaucoma by addressing limitations of previous work done to investigate astrocyte mechanobiology. For example, an overwhelming majority of astrocyte mechanobiology research has been conducted using 2D monolayer culture systems<sup>8</sup>. These are problematic *in vitro* models because they induce astrocyte activation in the absence of

mechanical stimulation and, therefore, cannot be used to assess the true nature of astrocyte reactivity to individual stimuli<sup>8,9</sup>. The limited research conducted using 3D culture systems has yielded comparatively smaller baseline activation levels, making them a better candidate for creating *in vivo* systems<sup>10,11</sup>. Unlike 2D culture systems, 3D systems can also model the main mode of biomechanical strain: compression<sup>10,13</sup>. Furthermore, most *in vitro* research exploring astrocyte mechanobiology has relied on the use of the commercially available DiTNC1 cell line. Although astrocytes, DiTNC1 cells are extracted from the neocortical layer of the brain<sup>13</sup> and may differ in cellular response and subsequent alteration of the biochemical environment in response to mechanical stressors compared to ONH astrocytes. Therefore, we propose to isolate astrocytes from the ONH and use them to investigate astrocyte mechanotransduction properties.

## **METHODS**

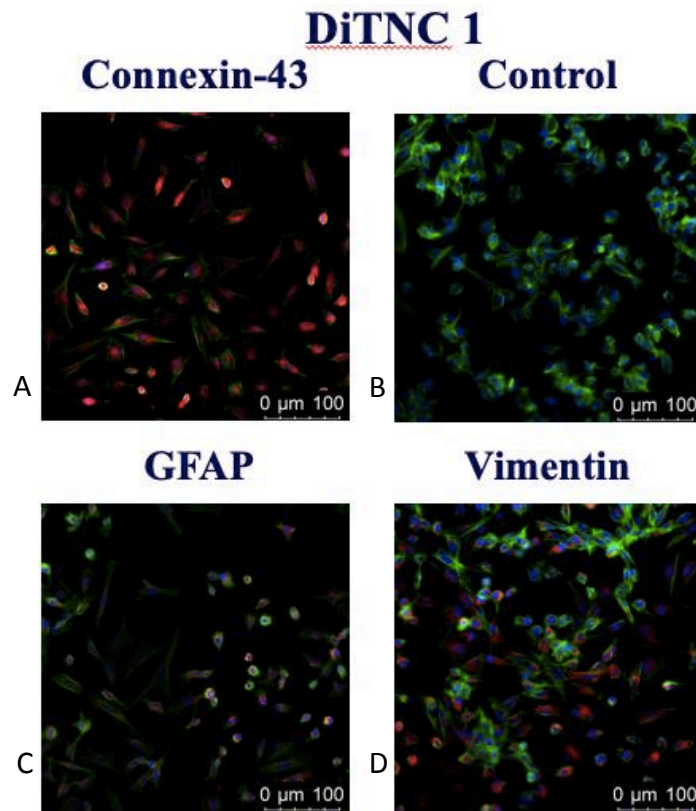
*Isolation of Rat ONH cells (Type 1A)* - Eyes were enucleated from 3 female CD rats, aged 6 months, and the optic nerve head was dissected out and broken down. Tissue was digested in 0.1% trypsin/EDTA, then centrifuged to isolate cells from tissue<sup>14</sup>.

*Cell Culture* - Cells were cultured in a growth medium (DMEM, 20% FBS, 1X pen/strep) and seeded on poly-L-lysine (PLL) coated coverslips. Coverslips were stored in PBS<sup>14</sup>.

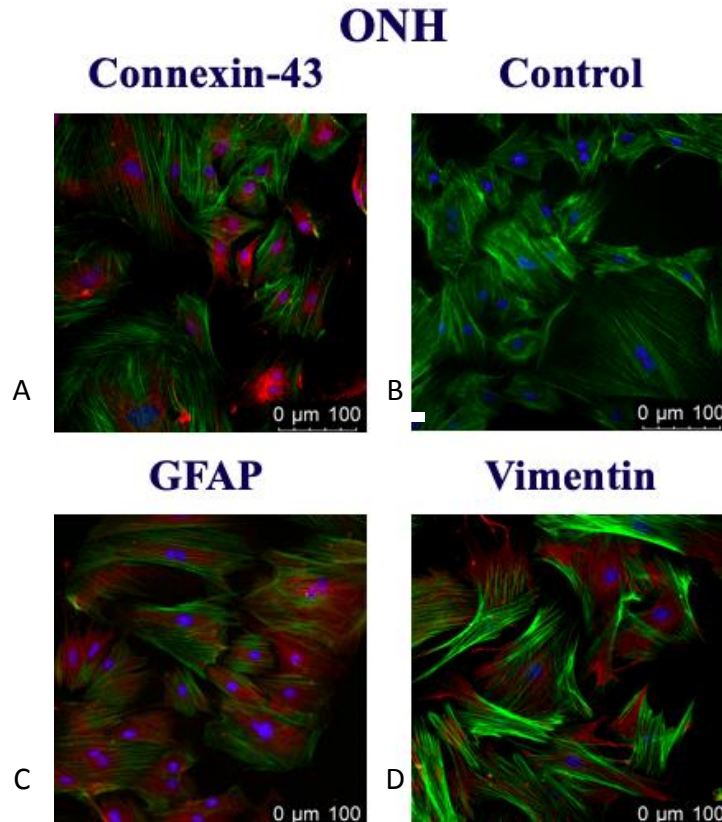
*Immunohistochemistry*- Cells on coverslips were fixed in paraformaldehyde for half an hour, then blocked in a blocking solution (10% FBS, 2% sodium azide, and 88% PBS) for an hour at room temperature. DiTNC1 cells were used as a positive control. Cells were washed twice with the blocking buffer and incubated in primary antibodies diluted in blocking buffer at 4°C overnight: Connexin-43 (Invitrogen, 1:50), GFAP (Invitrogen, 1:200), and Vimentin (1:200, Santa Cruz Biotechnology). Controls were left in blocking buffer. Cells were then washed 3 times with blocking buffer, leaving 10 minutes between each wash, and placed in secondary

antibody solution consisting of Phalloidin (1:30, Abcam), Alexa Fluor®647 anti-mouse or anti-rabbit IgG (1:200, Molecular Probes-Invitrogen), and DAPI (3 drops, Molecular Probes) diluted in blocking solution for an hour at room temperature in the dark. Controls for both cell types were prepared with secondary treatment only. Cells were washed two times with blocking solution, leaving 10 minutes between each wash, mounted, and imaged using a Leica DM6 B microscope. The same gain settings were used for each set of control and experimental samples to ensure consistency and validity when evaluating protein presence.

## RESULTS



**Figure 1:** Immunofluorescence labeling of DiTNC1 cells. (A, C-D) Immunolabeling for connexin-43, GFAP, and vimentin (*red*), respectively, merged with phalloidin (*green*) and nuclei (*blue*) staining. (B) Immunolabeling for phalloidin and nuclei only. Positive staining was present for all three astrocyte markers.



**Figure 2:** Immunofluorescence labeling of primary ONH cells. (A, C-D) Immunolabeling for connexin-43, GFAP, and vimentin (*red*), respectively, merged with phalloidin (*green*) and nuclei (*blue*) staining. (B) Immunolabeling for phalloidin and nuclei only. Positive staining was present for all three astrocyte markers.

## DISCUSSION

Positive staining for the three astrocyte markers connexin-43, glial fibrillary acidic protein, and vimentin allowed us to verify the cell type. Differences in cell morphology between DiTNC1 cells and primary ONH astrocytes suggests that significant differences exist between the cell types, which may be important in studies investigating how astrocyte mechanobiology impacts progression of glaucoma. The large size of the ONH astrocytes complicates development of a model *in vivo* system because formation of large spheroids increases

heterogeneity of cell stages. Additionally, presence of spheroids in hydrogels may hinder mimicry of *in vivo* vascularization because it is more difficult for substances to move into larger spheroids via diffusion, which is the only mode of cell entry in 3D culture systems<sup>18</sup>.

## **CONCLUSIONS & FUTURE WORK**

Now that we have isolated primary ONH astrocytes, we can begin testing of our 3D culture system. We will need to verify primary astrocyte viability in the 3D culture system as well as analyse the construct for hypoxia. Members of the Ethier lab have developed a bioreactor capable of applying uniform cyclic compression to the hydrogels, modelling the main form of biomechanical strain that the ONH experiences. Afterwards, the mechanical properties of the 3D hydrogel constructs will need to be assessed using unconfined compression testing to ensure the matrix environment is mechanically similar to an ONH environment. Once a physiologically representative environment in the construct has been confirmed, we can direct efforts towards understanding astrocyte responses to mechanical forces. Understanding the pathway will allow us to explore compounds that interfere with astrocyte mechanotransduction such as nonselective P2 receptor antagonists, which have been shown to impede crucial communication between astrocytes and neurons. We can also evaluate mechanically stimulated astrocytes for expression of proteins like nestin, tumor necrosis factors, and matrix metalloproteinases that are known to play a role in remodelling the extracellular matrix surrounding RGCs<sup>6,15, 16</sup>. Additionally, our system will diminish the need for animal subjects to study glaucoma, and serve as a step towards the creation of an “optic nerve head on a chip” to offer patients a permanent solution to glaucoma.

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