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Title
Labeling Tight Junction Proteins ZO1, Occludin, Claudin 3, and Claudin 4 in T84 Intestinal Epithelial Cells

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LABELING TIGHT JUNCTION PROTEINS

ZO1, OCCLUDIN, CLAUDIN 3, AND CLAUDIN 4

IN T84 INTESTINAL EPITHELIAL CELLS

By

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A capstone project submitted for
Graduation with University Honors

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University of California, Riverside

APPROVED

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Dr. Richard Cardullo, Howard H Hays Jr. Chair, University Honors

Abstract

As the central nervous system's (CNS) resident immune cells, microglia help maintain the brain as well as facilitate its defenses and repair. Recent research has shown that defects in microglia can have consequences on overall brain health and can contribute to the onset and progression of neurodegenerative diseases and neurocognitive disorders. These defects can be caused by different factors such as age, genetics, pathogens, and the environment. Recent research has also shown that gut-brain interactions and communications can have an impact on the pathogenesis of several neurological disorders. By performing single labels of tight junction proteins ZO1, Occludin, Claudin 3, and Claudin 4 in T84 intestinal epithelial cells, we will be able to perform double labels of said proteins in future gut-brain and blood brain barrier interaction experiments and improve efficiency of these experiments by reducing the amount of materials needed in comparison to performing multiple single labels.

Acknowledgments

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Introduction

Microglia are the central nervous system's resident macrophages, and they are quite sensitive to brain disease and injury. Microglia adopt an activated form by changing their structure in response to brain damage, and the structure is ultimately altered by glial inflammation. This inflammation has been shown to increase the progression and onset of neurodegenerative diseases (1). Along with glial inflammation, disruption of the blood brain barrier that leads to blood to brain extravasation of neuroinflammatory molecules may also cause an increased risk is neurodegenerative diseases. Increased blood brain barrier permeability, which is what can cause blood to brain extravasation, has been shown to decrease tight junction protein expression in aged rodents (3), and there is evidence showing that the expression of tight junction proteins is impacted by activated microglia (6). Blood brain barrier impairment has also been shown to be a factor contributing to Alzheimer's disease, since the barrier maintains brain homeostasis, and with Alzheimer's, the loss of tight junction proteins is common (4). The purpose of my project is to culture T84 cells and achieve successful labels for their tight junction proteins ZO1, Occludin, Claudin 3, and Claudin 4. T84 cells are a cancer cell line and were collected from the lung metastasis of a 72 year old male's colon carcinoma (5). By establishing proper protocols for the single labeling of these proteins, double labels may be performed in future experiments that further involve the impact of tight junction proteins on the blood brain barrier and therefore on overall brain health. With double labels, more data may be collected with less resources, making the overall experiment more efficient.

Methods and Materials

Labeling tight junction proteins requires a few different protocols, some of which had to be changed in order to yield optimal results. To begin the process, T84 cells were maintained in a culture flask using advanced DMEM F12 media mixed with 2% Fetal Bovine Serum, 1% glutamate and 1% penicillin and streptomycin. When the cells reached an adequate confluency, the protocol necessary to culture the cells in a 24-well plate was performed. Around six to seven wells were used per plate, and each well had cover slips previously incubated in Poly-D-Lysine (PDL). PDL helps the cells attach to the cover slips, and the cover slips are required to visualize the cells per microscope later on in the experiment. A hemocytometer was used to count the cells present in the culture flask in order to dilute the cells accordingly with media to make sure that around 500,000 were present in each well of the plate. Once these dilutions were performed and the cells were inputted into the 24 well plates, the plates were cultured for a week with regular media changes. After a week, the cells proved confluent enough to experiment on. The cells were fixed with paraformaldehyde or methanol, and then incubated with the desired dilution of the primary antibody (ZO1, Occludin, Claudin 3, or Claudin 4) for 24 hours. The cells were then incubated with an anti-mouse 488 or 594 or an anti-rabbit 488 or 594 secondary antibody for 24 hours. Keeping double labels in mind, it is important to note that it is vital for secondary antibodies to not cross react, meaning they cannot be of the same wavelength or against the same species of animal, to be able to visualize the desired tight junction proteins separately. As Claudin 4 was the only anti-mouse protein, it was important to get a proper single label of the protein to have the opportunity to successfully perform a double label with an

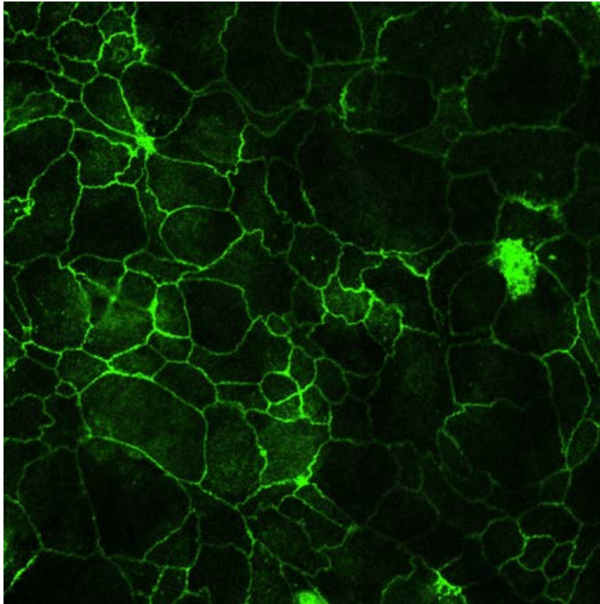
anti-rabbit protein (ZO1, Occludin, or Claudin 3) in the future. After the 24 hour secondary antibody incubation, the cover slips were incubated with DAPI, which labels cell nuclei, for 24 hours. Finally, the cover slips were put onto microscope slides to image the cells to see if the labeling was successful. It took several different combinations of working primary and secondary antibodies, cell fixation methods, and antibody dilutions to produce successful labeling for each protein.

Results

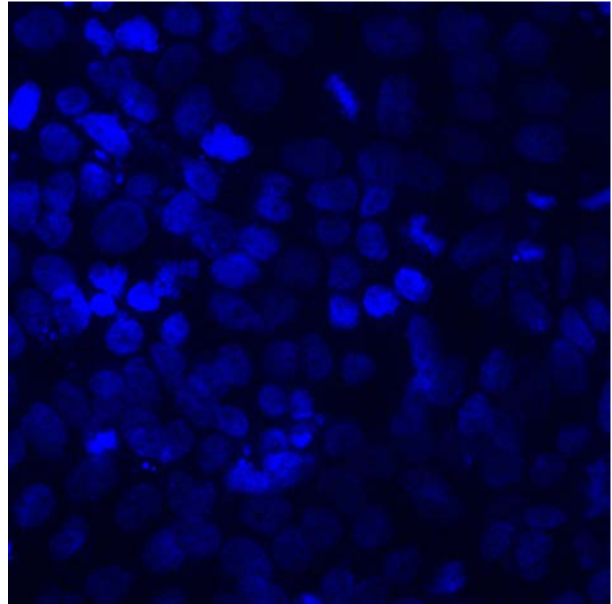
ZO1: This protein was labeled with a Rabbit 488 secondary antibody and later a Rabbit 594 antibody to make sure both secondary antibodies were viable. Both labels were a 1:100 dilution, were incubated with Poly-D-Lysine (PDL), and involved a paraformaldehyde (PFA) cell fixation.

Rabbit 488 Label

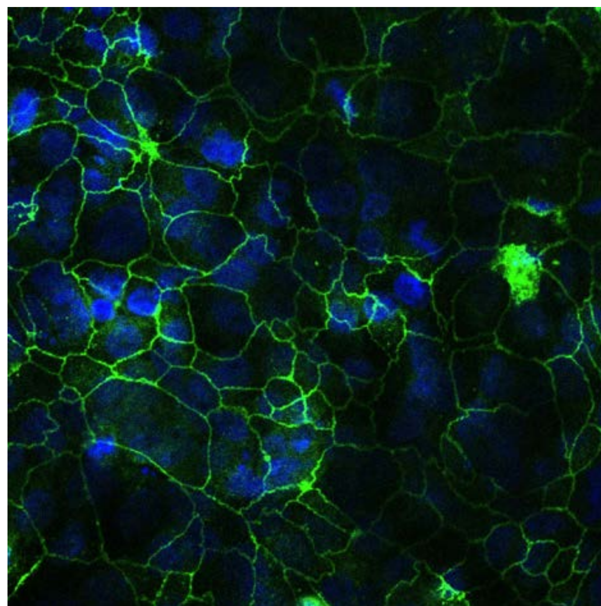
Tight Junction



DAPI (nuclei label)



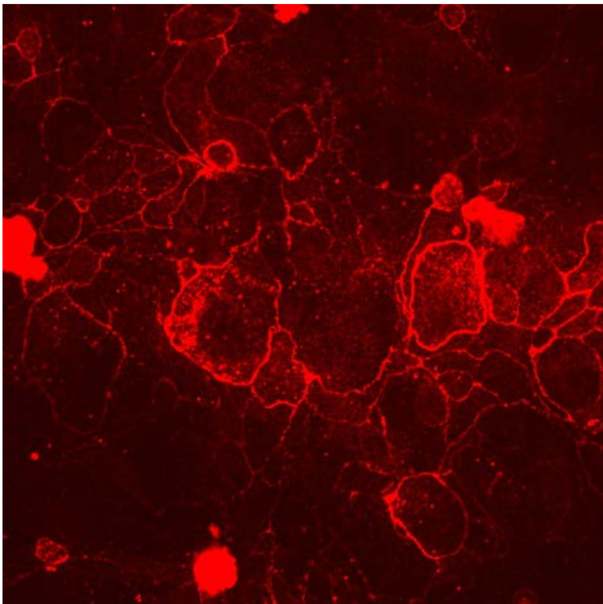
Tight Junction + DAPI

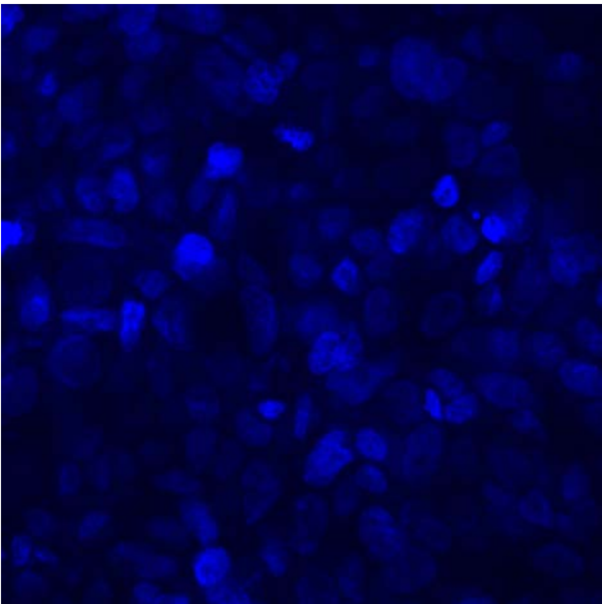


Rabbit 594 Label

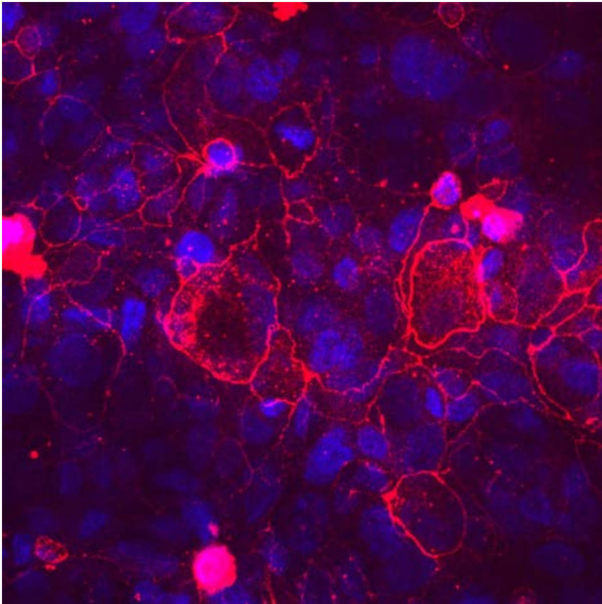
Tight Junction

DAPI





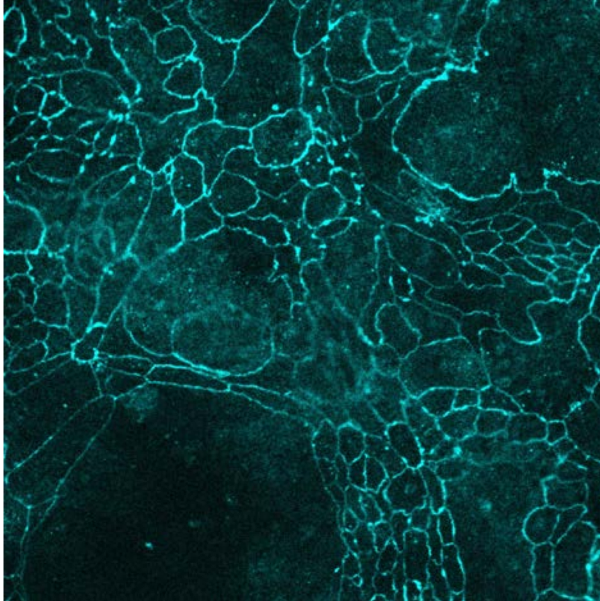
Tight Junction + DAPI



Occludin: This protein was labeled with a Rabbit 488 secondary antibody at a 1:200 dilution. The first label performed was with PDL incubation and PFA fixation. The second label was without PDL incubation and with methanol fixation.

PDL Incubation and PFA fixation

Tight Junction



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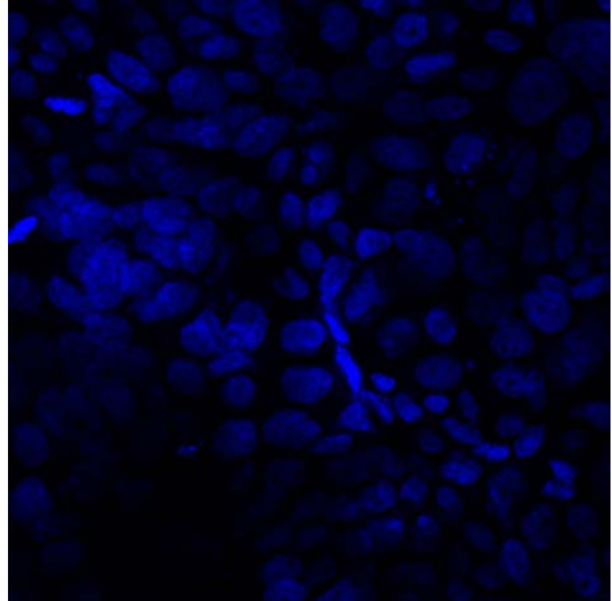
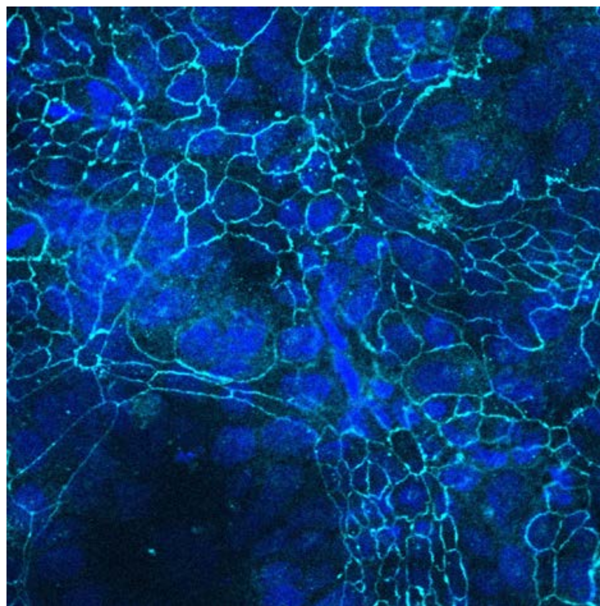
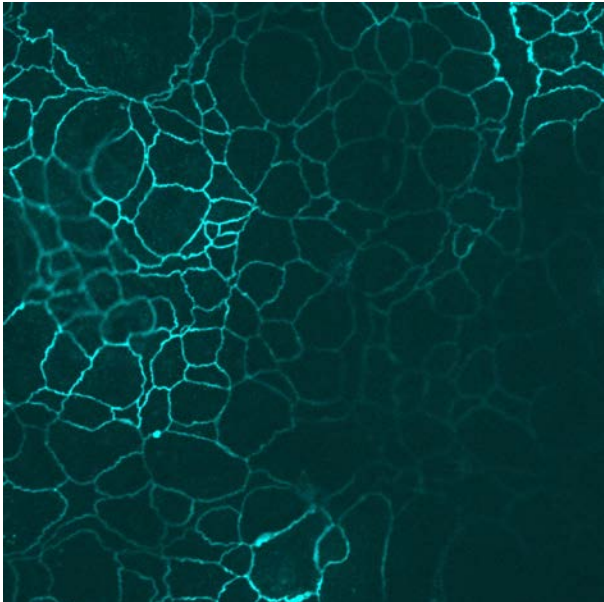


Tight Junction + DAPI



No PDL Incubation and Methanol Fixation

Tight Junction



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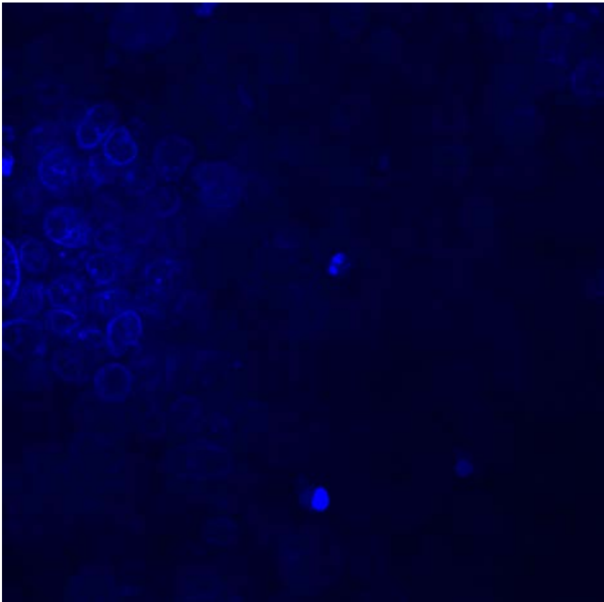
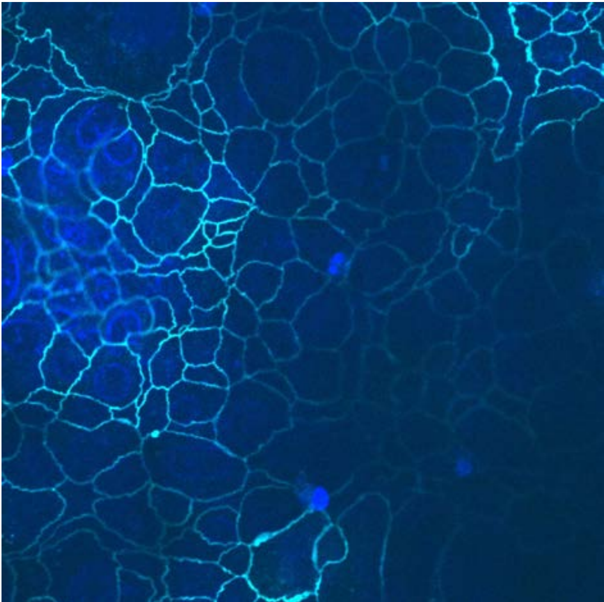
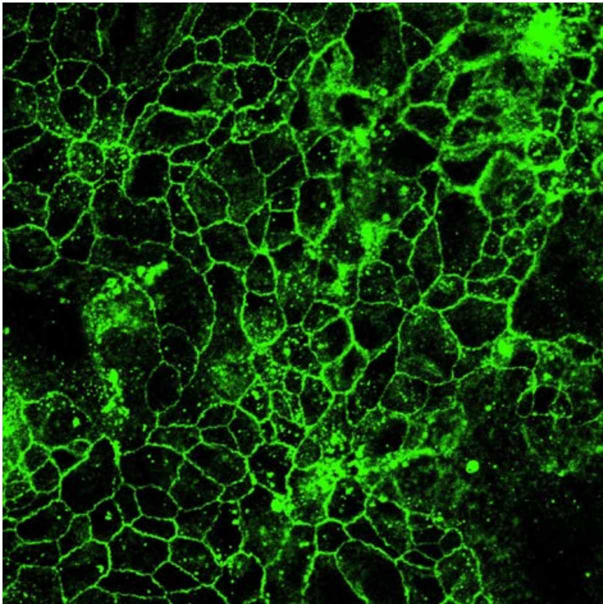


Tight Junction + DAPI



Claudin 3: This protein was labeled with a Rabbit 488 secondary antibody at a 1:100 dilution. This label involved PDL incubation and PFA fixation.

Tight Junction



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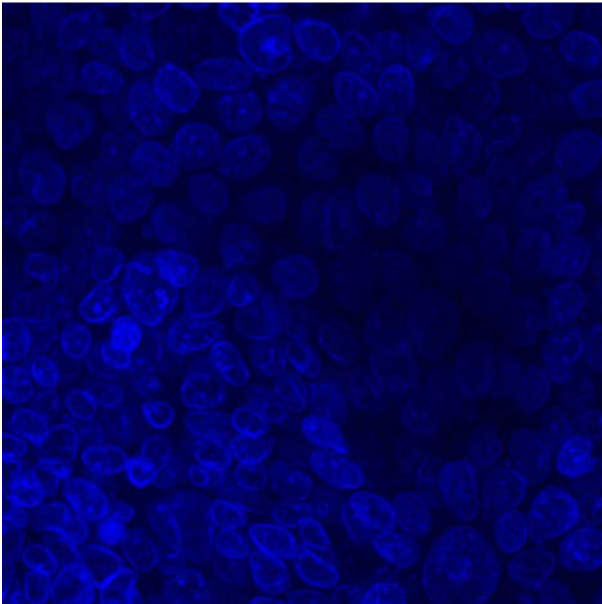
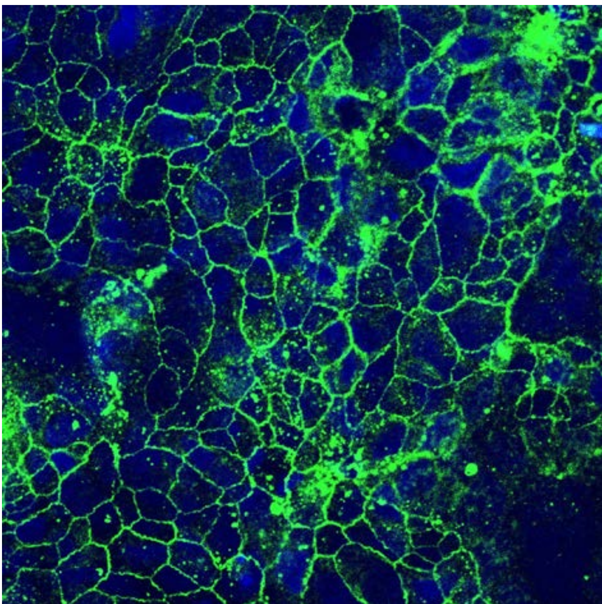


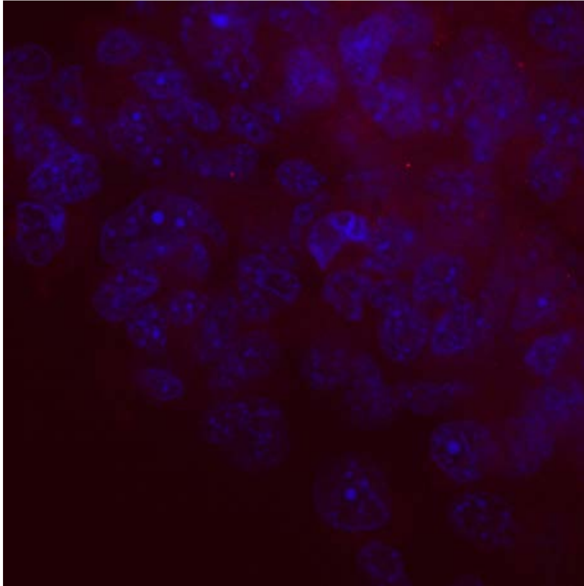
Tight Junction + DAPI



Claudin 4: This protein was the most difficult to label and involved a few unsuccessful labels. Primary antibody dilutions, the physical primary antibody, and fixation methods were all changed to finally produce a successful label.

Unsuccessful Labels: Nonspecific staining

Plate one consisted of a 1:50 and 1:100 dilution, respectively, with PFA fixation, PDL incubation, and a Mouse 594 secondary antibody.



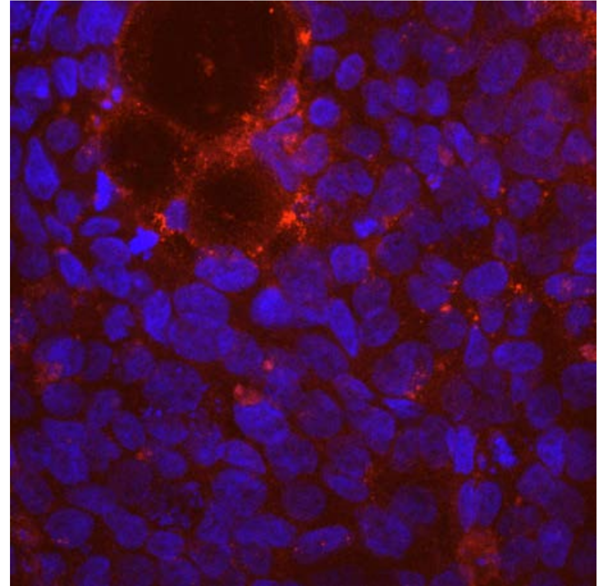
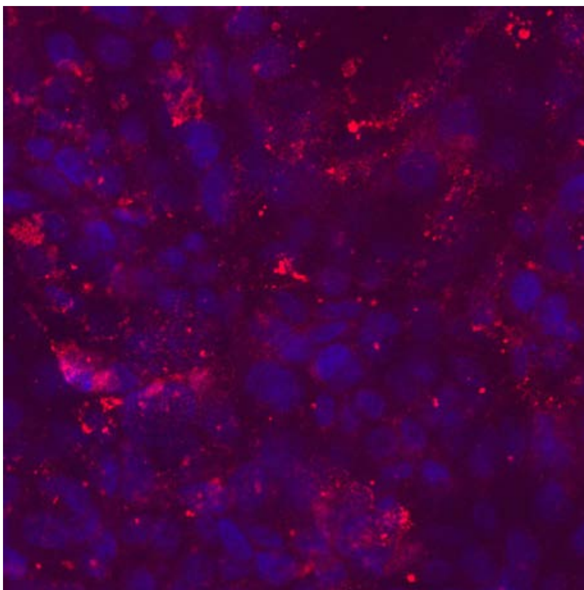


Plate two consisted of a 1:25 and 1:50 dilution, respectively, with methanol fixation, PDL incubation, and a mouse 488 secondary antibody



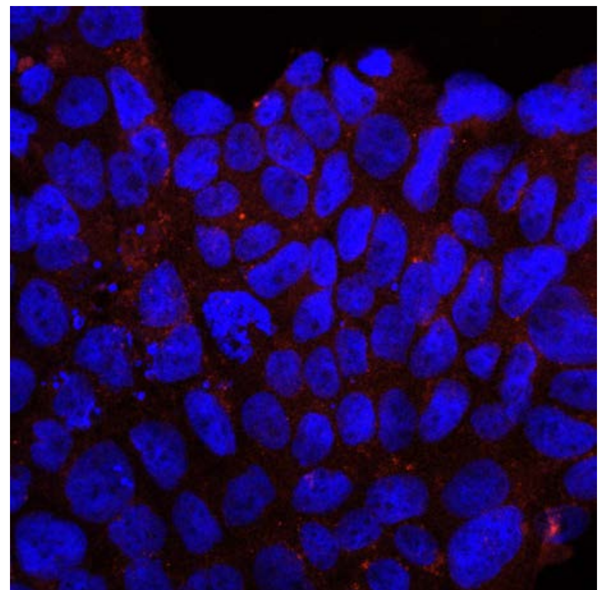
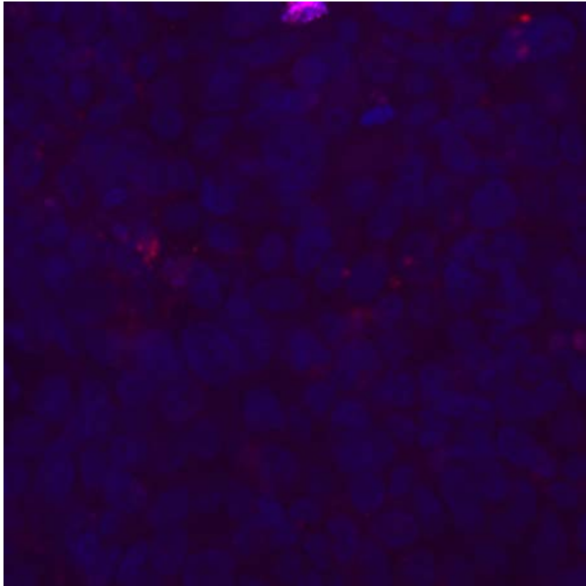
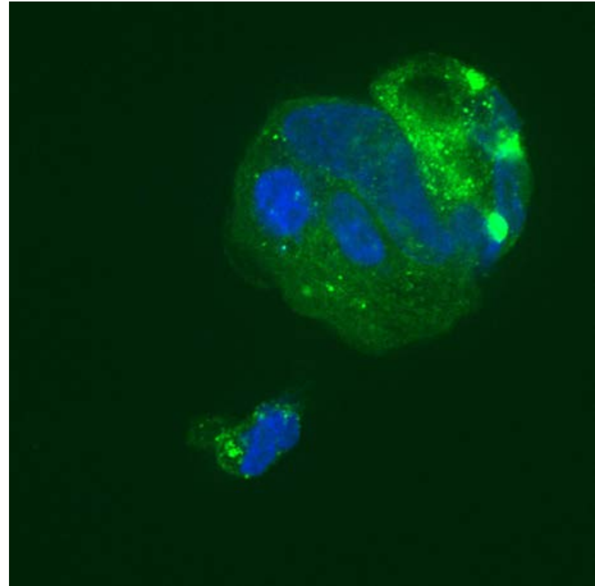


Plate three consisted of a 1:50 and 1:100 dilution with PFA fixation, Poly-D incubation, a Mouse 594 secondary antibody, and a new primary antibody.

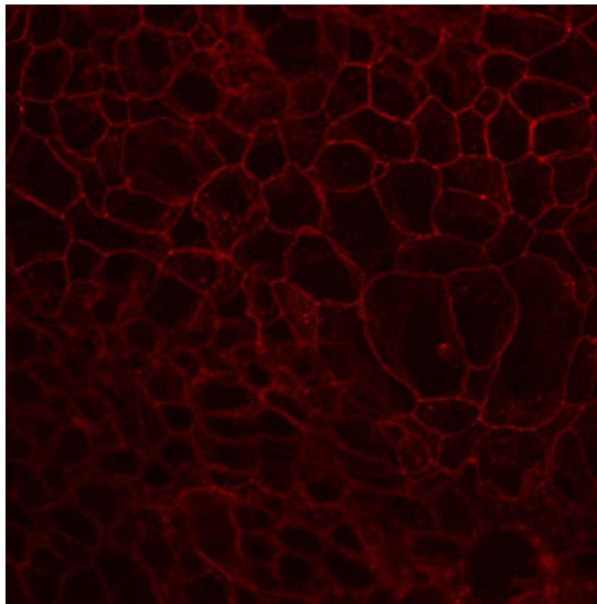




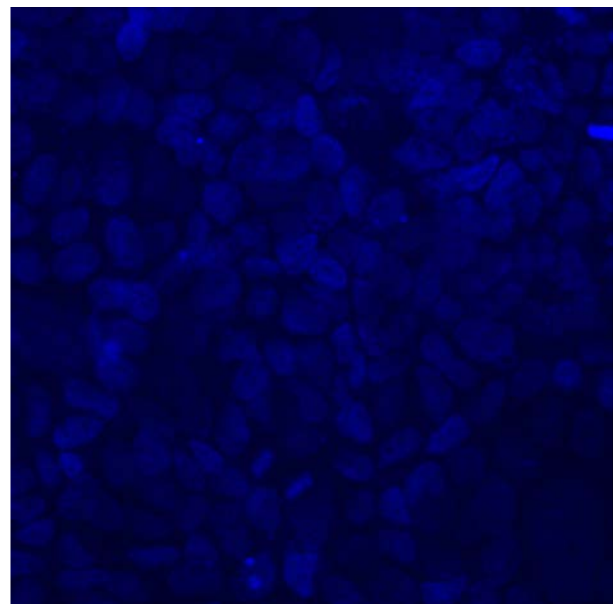
Successful Label

Plate four finally produced a successful label. It involved a 1:100 dilution, PDL incubation, a Mouse 594 secondary antibody, a new primary antibody, and methanol cell fixation.

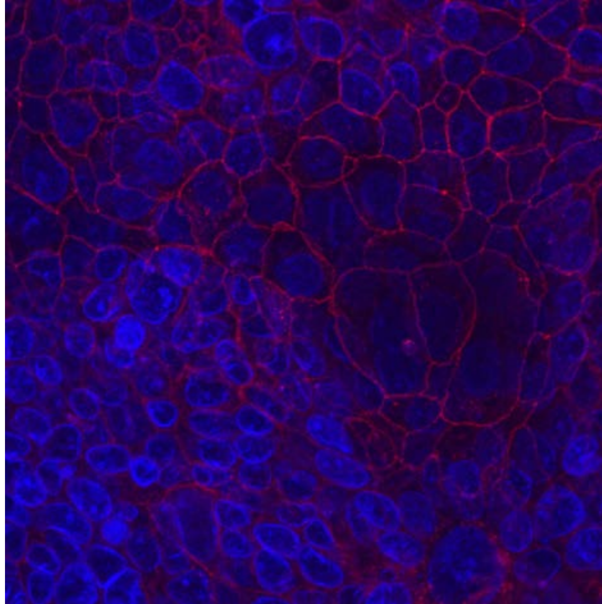
Tight Junction



DAPI



Tight Junction + DAPI



Conclusion

Successful single labels of all four of the tight junction proteins ZO1, Occludin, Claudin 3, and Claudin 4 were achieved. When a plate produced unsuccessful labels, variables such as different vials of primary and secondary antibodies, primary antibody dilutions, and fixation methods were altered. It was also seen that cover slip incubation with Poly-D-Lysine was not a necessarily vital step to get the cells to attach to the cover slip. However, it is still regarded as a good measure to take to make sure the labeling goes as smoothly as possible. Claudin 4 was the most difficult label to achieve, as it took three unsuccessful plates to finally image a working label. Changing the Claudin 4 fixation method from paraformaldehyde to methanol seemed to be the solution to get a successful label. Methanol fixation was also attempted on Occludin to see if it would be a valid protocol for other tight junction proteins as well. The table below shows all the successful labels produced taking into account all the variables that were changed.

Tight Junction Protein	Secondary Antibody	Fixation Method	Dilution
ZO1	Rabbit 488 and 594	Paraformaldehyde	1:100
Occludin	Rabbit 488	Paraformaldehyde and Methanol	1:200
Claudin 3	Rabbit 488	Paraformaldehyde	1:100
Claudin 4	Mouse 594	Methanol	1:100

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