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Method Article

Whole mount staining of lenses for visualization of lens epithelial cell proteins ☆

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A B S T R A C T

Whole mount imaging of the lens allows for high spatial resolution visualization of lens epithelial structures by using small molecule fluorescent probes. However, the visualization of specific proteins in lens epithelial cells within whole lenses remains a challenge as the capsule that surrounds the lens does not allow penetration of antibodies. Here we describe a whole mount imaging method that allows us to overcome this challenge by digesting the lens capsules of paraformaldehyde fixed lenses using collagenase. This method enables the penetration of antibodies for effective visualization of proteins in the epithelium of whole lenses.

- A limitation to lens whole mount imaging is the ability to visualize specific proteins as the collagen capsule surrounding the lens impedes the penetration of antibodies
- This protocol helps overcome this limitation by a light collagenase digestion of the capsule of fixed lenses prior to immunostaining
- This method allows for the imaging of specific proteins in the epithelium of the whole lens tissue

A R T I C L E I N F O
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Specifications Table

Subject Area	Biochemistry, Genetics and Molecular Biology
More specific subject area	Lens research
Method name	Whole Mount immunostaining of the lens in the eye
Name and reference of original method	*Tropomyosin 3.1 Association with Actin Stress Fibers is Required for Lens Epithelial to Mesenchymal Transition* *Parreno J, Amadeo MB, Kwon EH, Fowler VM* *Invest Opthalmol Vis Sci. 2020 Jun 3;61(6):2*
Resource availability	

Method details

Background

The ocular lens is a transparent tissue that fine focuses light onto the retina. With exception to the thin collagenous capsule that surrounds the lens [1,2], the lens is predominantly cellular and consists of two main cell types: fiber and epithelial cells. The fiber cells make up a bulk of the lens while the epithelial cells form a monolayer in its anterior region [3]. The lens epithelial cells continually proliferate and differentiate into fiber cells to support lens growth, a process that occurs throughout life. However, failure to maintain homeostasis in this anterior region of the lens could result in epithelial to mesenchymal transition (EMT) and pathologies, such as anterior subcapsular or posterior capsular opacification [1,4–14]. Visualizing proteins in lens epithelial cells within intact lenses will help understand lens homeostasis as well as pathological processes.

The *ex vivo* culture of intact, isolated lenses is a powerful method to study lens epithelial cell biology and pathology [7,15,16]. As compared to two-dimensional culture of lens epithelial derived cells or cell lines, whole lens culture allows for preservation of lens/cellular structures. In intact lenses, epithelial cells remain attached to each other—to the capsule at the basal regions and to the fiber cells at the apical regions. Additionally, whole tissue culture of lenses has allowed for the emergence of lens mechanobiology. We demonstrated how tissue-scale mechanical loads are transmitted onto lens epithelial cells [15,16]; moreover, others have demonstrated that whole lens stretch results in lens epithelial cell proliferation [16]. Furthermore, the *ex vivo* culture system has allowed for the study of lens EMT [4,5,7,17–19]. By culturing lenses in the presence of TGFβ, the lens EMT process (i.e., lens fibrosis) can be initiated and comprehensively examined. The use of whole lens cultures is an ideal model to mimic *in vivo* physiological and pathological conditions.

A challenge in using whole lenses for experimentation is whole mount imaging of specific proteins in lens epithelial cells. While visualization of specific proteins in lens cells can be achieved by isolation of lens cells, flat mounting of the lens epithelium, or tissue sectioning [20–23], these methods disrupt overall tissue architecture. Therefore, new methodology to stain specific proteins in lens epithelial cells within whole lenses is required. While the capsule allows the penetration of small fluorescent chemical probes (i.e., phalloidin-conjugates to image F-actin) [24], the penetration of antibodies, which are larger than chemical probes, is restricted. To enable whole lens epithelial immunostaining, we utilize a collagenase digestion of fixed whole lenses. Since the lens capsule is predominantly collagen [1], our rationale is that gentle capsular digestion using collagenase would permit the penetration of antibodies to enable immunostaining of lens epithelial cells in whole lenses. Here we describe a methodology, that we have previously developed [4], that enables immunofluorescent staining for confocal microscopy providing high spatial resolution imaging of specific proteins within epithelial cells.

Materials and methods

Mice and lens dissections

All procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement on the Use of Animals in Ophthalmic and Vision Research and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. Mouse care

and euthanasia procedures were approved by the University of Delaware Institutional Animal Care and Use Committee (IACUC).

Eyes were enucleated from wild-type C57BL/6 mice 8–10 weeks of age. Lenses were dissected in 1X phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH8.1) as previously described [15].

Solution/Buffer preparation (source of materials are listed in Table 1)

1. **4% paraformaldehyde (PFA) in PBS:** Dilute 16% PFA in 10X PBS and deionized water to get a final concentration of 4% PFA in 1X PBS
2. **Collagenase solution:** Prepare 0.25% collagenase A in 1X PBS
3. **Permeabilization/Blocking Buffer:** Mix 3% goat serum + 0.3% Bovine serum albumin + 0.3% Triton X-100 in 1X PBS
4. **Primary (1°) Antibody Solution:** Dilute 1° antibody in 200 µl of Permeabilization/Blocking Buffer. For method validation we use a 1:100 dilution of E-cadherin antibody.
5. **Secondary (2°) Antibody Solution:** Dilute Hoechst (1:500 dilution), Rhodamine-Phalloidin (at 1:20 dilution), and fluorescent conjugated 2° antibody in 200 µL of Permeabilization/Blocking Buffer. For method validation, we use a 1:100 dilution of Goat anti Rabbit Alexa 488.

Table 1.
Materials required in protocol.

Materials/Reagent	Manufacturer	Part/Catalog #
Suspension Culture Plates (non-treated)	Genesee Scientific	25-103
Embryonic forceps	Hammacher Instrumente	HSC 702-93
48-well plate	Genesee Scientific	25-103
Paraformaldehyde (16%)	Electron Microscopy Sciences	15710
DPBS (10x, without Ca, Mg)	Gibco	14200-075
PBS (1x, without Ca, Mg, Phenol Red)	Genesee Scientific	25-507
Collagenase A	Roche	10103586001
Goat Serum	Invitrogen	A11008
Bovine Serum Albumin	Genesee Scientific	25-529
Triton™ X-100	Thermo Scientific	28314
ProLong™ Gold Antifade Mountant	Invitrogen	P36934
35mm Dish (No. 1.5 coverslip, 14mm	MatTek Life Sciences	P35G-1.5-14-C
Agarose	Genesee Scientific	20-101
Biopsy punch (2mm)	Acu-punch	P225

Staining procedure

This procedure is conducted in a non-tissue culture treated 48 well plate. The materials used and sources of materials are listed in Table 1. Individual lenses are incubated within wells consisting of solutions (Figure 1) for the durations listed in Table 2. Lenses are sequentially transferred, after a given time, into subsequent wells using embryonic forceps to prevent lens damage.

1. Fix the freshly dissected or cultured lenses in 4% PFA at room temperature for 30 min.
2. Wash lenses by transferring them into wells consisting of 500 µL of 1X PBS. Repeat 3 times for 5 min per wash.
3. Transfer lenses into wells that consist of 200µl of 0.25% collagenase solution. Incubate in collagenase solution at 37 °C for 30 min.
4. Transfer lenses into wells consisting of 200 µl of Permeabilization/Blocking Buffer and incubate at room temperature for 2 h.
5. Place lenses in 1° antibody solution. Incubate at 4 °C overnight.
6. Wash lenses 6 times by transferring them into wells containing 500 µl of 1X PBS.
7. Transfer lenses into 2° antibody solution and incubate at room temperature for 2 h in a dark setting (i.e., cover plate with foil).
8. Wash lenses 6 times by transferring them into wells containing 500 µl of 1X PBS.
9. Image lenses on confocal microscope. If not imaging immediately, place lenses in 80 µL 1X PBS with 1 drop of ProLong Gold antifade reagent.

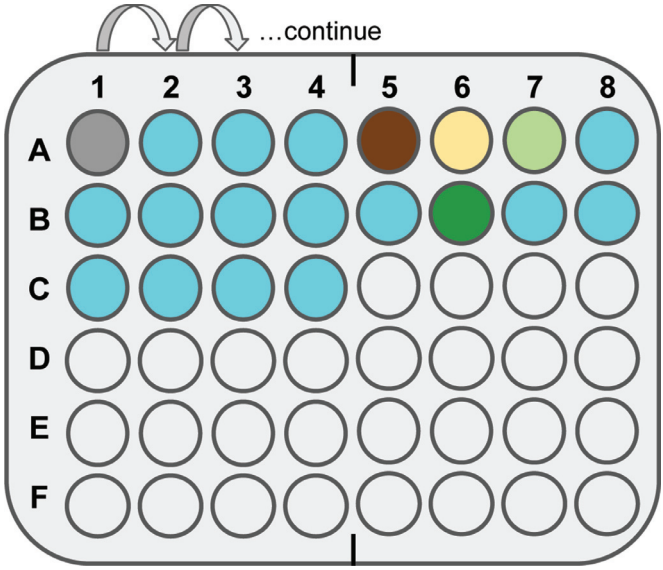

Fig. 1. Schematic of staining procedure in a 48-well plate. The solutions, and the duration of lens incubation, in each well are indicated in Table 2.

Table 2.
Step by step summary for Whole Mount Lens immunofluorescence staining protocol

Step #	Incubation Condition	Well	Reagent	Color	Volume	Temperature	Time Per Well
1	Fixation	A1	4% PFA	Gray	500ul	RT	30 mins
2	Washes	A2-A4	1X PBS	Blue	500ul	RT	5 mins each
3	Capsule Digestion	A5	0.1% Collagenase	Brown	200ul	37°C	1 hour
4	Permeabilization/ Blocking	A6	Permeabilization/ Blocking Buffer	Yellow	200ul	RT	2 hours
5	1° Antibody	A7	1° Antibody solution	Light Green	200ul	4°C	Overnight (14-18 hours)
6A	Washes	A8, B2, B4	1X PBS	Blue	500ul	RT	Brief washes ~10 seconds each
6B	Washes	B1, B3, B5	1X PBS	Blue	500ul	RT	5 mins each
7	2° Antibody	B6	2° Antibody solution	Dark Green	200ul	RT	2 hours
8A	Washes	B7, C1, C3	1X PBS	Blue	500ul	RT	Brief washes ~10 seconds each
8B	Washes	B8, C2, C4	1X PBS	Blue	500ul	RT	5 mins each

Stabilizing Lens within Agarose Divot for Confocal Imaging:

1. To stabilize lenses during imaging, we immobilize lenses on a glass dish within an agarose divot (Fig. 2). To create the divot, using a 2 ml disposable graduated transfer pipette, place molten 2% agarose in 1X PBS on the center of a glass bottom dish. Drop a flexible plastic coverslip to flatten the agarose. Once the agarose has gelled, remove coverslip and then punch a 2 mm hole into the center of the agarose using a biopsy punch. Rinse in 1X PBS and remove excess agarose by vacuum aspiration.
2. Put 2 mL of 1X PBS into the dish and place the lens into the divot. The lens should be completely submerged, and lens movement should be restricted to within the divot.
3. Finally, under the confocal microscope, confirm that the anterior side of the lens is facing the microscope objective. This is indicated by examining nuclear staining (Hoechst) as lens

epithelial cells are present in the anterior but not the posterior region of the lens. If nuclei are not visible, this indicates the posterior region is facing the microscope objective. Using curved forceps, gently flip the lens over onto the anterior side.

4. Once the anterior region of the lens is facing the objective, place a glass coverslip on the posterior region of the lens to further restrict movement.
5. Image the lens epithelial cells.

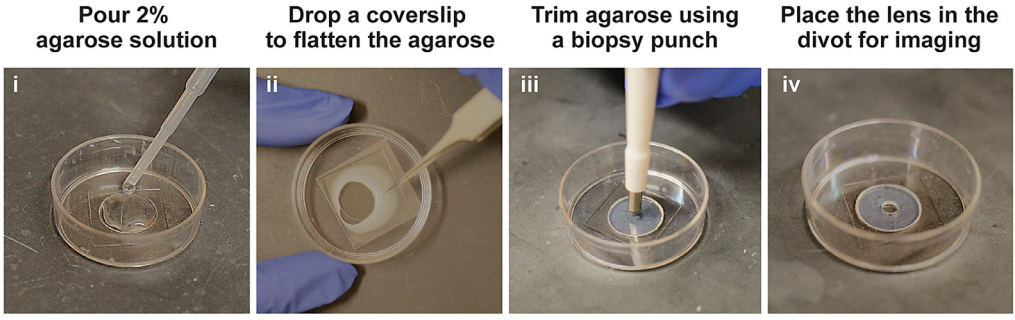

Fig. 2. To create agarose divot (i) pour molten 2% agarose in a glass bottom dish. Next, (ii) flatten the agarose using a flexible plastic coverslip. (iii) Using a biopsy punch, make a divot within the gelled agarose. (iv) Keep the lenses within divot.

Method validation

F-actin can be visualized by staining lenses with rhodamine-phalloidin in both non-collagenase and collagenase treated lenses (Fig. 3). However, the visualization of lens epithelial cell-cell junction

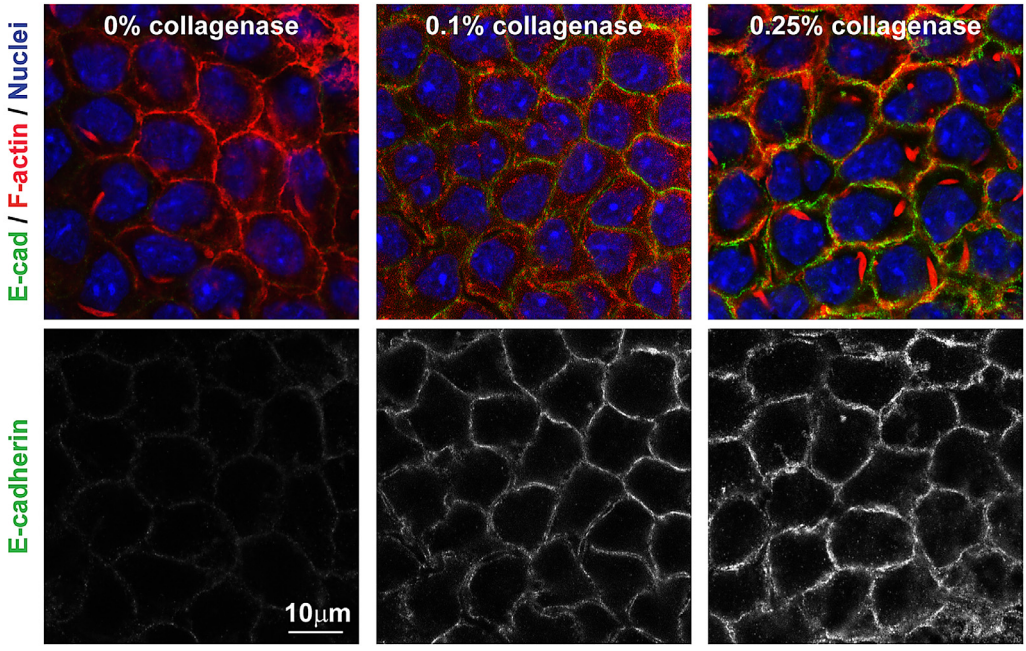

Fig. 3. Confocal microscopy images of lens epithelial cells of whole mounted lens. While F-actin (rhodamine-phalloidin; red) can be visualized in both non-collagenase and collagenase treated lenses, E-cadherin (green) is only visible in lenses where capsules are digested with collagenase. Treatment of fixed lenses with 0.25% collagenase is optimal for the visualization of E-cadherin in lenses from mice between the ages of 10 weeks. Nuclei counterstained with Hoechst 33342 (blue). Scale bar = 10 μm. Images were taken on a Zeiss 880 confocal microscope using a 63x (1.4NA) objective with a 2.5X zoom and were processed using Zen Black 2.3SP1 (Zeiss) software.

protein E-cadherin at the cell membranes by indirect immunofluorescence, is restricted in non-collagenase treated lenses. By treating lenses with 0.1% or 0.25% collagenase prior to immunostaining, E-cadherin is visible, with greater staining in lenses treated with 0.25% collagenase as compared to 0.1% collagenase. Additionally, visualization of ß-actin is restricted in non-collagenase treated lenses. By treating fixed lenses with 0.25% collagenase prior to immunostaining, ß-actin protein can be visualized as shown by staining at the basal and lateral regions of epithelial cells (Fig. 4a). Furthermore, we determined that our protocol also allows for visualization of β-actin in peripheral most fiber cells at the equatorial region of the lens. (Fig. 4b).

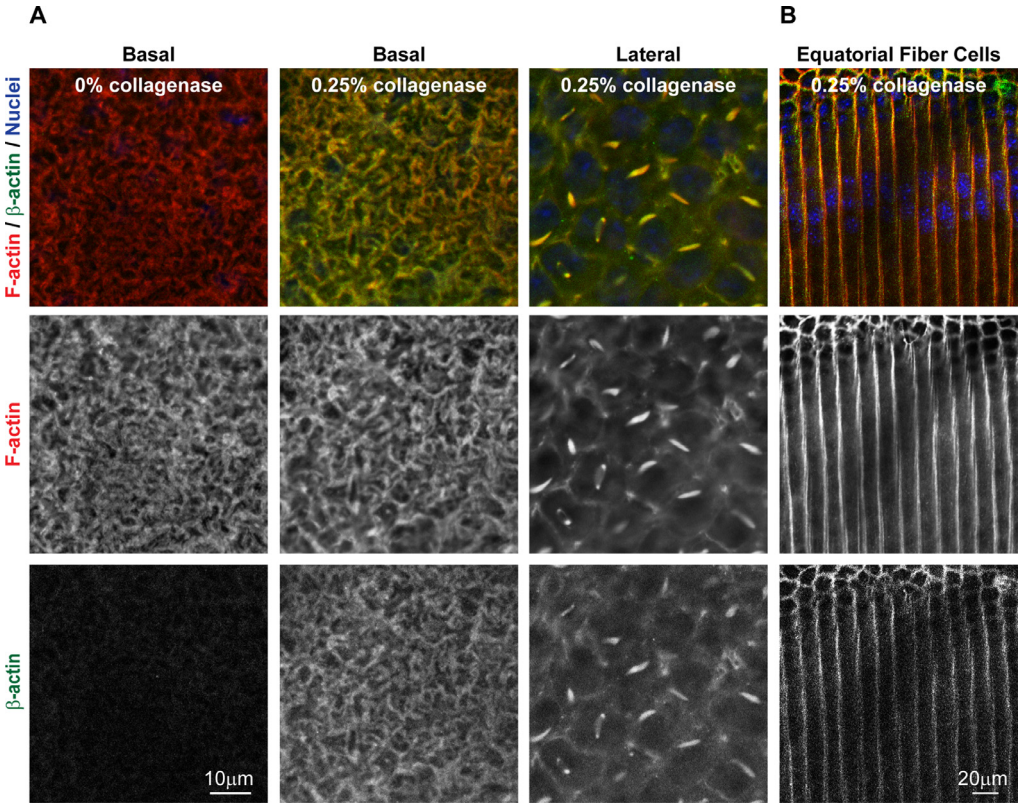

Fig. 4. Confocal microscopy images of lens cells in whole mounted lens. (A) While F-actin (rhodamine-phalloidin; red) can be visualized in both non-collagenase and collagenase treated lenses, ß-actin (green) is barely visible in whole mounted lens cells. Treatment of fixed lenses with 0.25% collagenase allows for the visualization of specific proteins within lens cells as shown by β-actin staining at the basal and lateral regions of epithelial cells. Nuclei counterstained with Hoescht 33342. Scale bar = 10 μm. Images were taken on a Zeiss 880 confocal microscope using a 63x (1.4NA) objective with a 2.5x zoom. (B) Treatment of fixed lenses with 0.25% collagenase also allows for the visualization of the ß-actin protein in the peripheral most fiber cell layers. Nuclei counterstained with Hoechst 33342 (blue). Scale bar = 20 μm. Images were taken on a Zeiss 880 confocal microscope using a 40x (1.3 NA) objective with a 1x zoom. Images were processed using Zen Black 2.3SP1 (Zeiss) software.

Conclusions

This protocol overcomes the difficulties associated with immunofluorescent staining of lens epithelial proteins within whole lenses. The conditions used in this protocol (0.25% collagenase at 37 °C for 30 min) are for mouse lenses isolated from 8–10-week-old C57BL/6 mice fixed in 4% paraformaldehyde. Lens capsule thickness is age dependent [25] and can vary between mouse strains [26], therefore staining conditions could vary when using lenses from different ages or

strains. Furthermore, we have not evaluated if this protocol can be used with other fixatives (i.e., methanol). In preliminary experiments, we have evaluated this protocol using different collagenase concentrations (0.1–1%) (Table 3). We found exposure of whole lenses to collagenase >0.25% led to excessive capsule matrix disruption resulting in loss of cellular organization. It may be possible to use higher concentration of collagenase by shortening incubation times < 30 min. While our methodology provides a basis that allows for effective visualization of proteins, conditions may need to be modified and determined empirically based on the specific needs of experimental samples/conditions. Finally, since collagenase enzymatically digests collagen in the lens capsule, this protocol is limited to staining cellular proteins and is not suitable for immunostaining of matrix proteins in the lens capsule.

Table 3
Collagenase Treatment Conditions.

Collagenase Concentration (%)	Time	Result
0%	30 min	Limited staining
0.10%	30 min	Staining
0.25%	30 min	Optimal staining
0.50%	30 min	Capsule disruption- loss of cellular organization
1%	30 min	Capsule disruption- loss of cellular organization

The method provided here can be used for investigating proteins that are expressed in lens epithelial cells. We have used this protocol previously [4] and have since used this protocol to image several proteins using the antibodies and dilutions listed in Table 4. While we have validated this method in the mouse lens, we anticipate the method can also be adapted to visualize specific proteins in the epithelium of other vertebrate lenses.

Table 4
Antibodies/dyes and their respective dilutions used for protein/probe detection.

Protein	Manufacturer	Catalog #	Dilution
E-cadherin	Cell Signaling	3195S	1:100
Beta-Actin	Millipore	MA1-91399	1:100
Tropomyosin 3.1/3.2 (Tpm3.1/3.2)	Millipore	Clone 2G10.2	1:100
Embryonic Lethal Abnormal Vision-Like1 (ElavL1/Hur)	Abcam	Ab200342	1:100
CUGBP Elav-Like Family Member 1 (Celf1)	Abcam	Ab9549	1:250
Alpha smooth muscle actin	Abcam	Ab7817	1:100
Goat anti rabbit IgG Alexa Fluor® 488	Invitrogen	A-11008	1:100
Goat anti mouse IgG Alexa Fluor® 488	Invitrogen	A-11001	1:100
Chicken anti rabbit IgG Alexa Fluor® 488	Invitrogen	A-21441	1:100
Hoechst 33342	Invitrogen	H3579	1:500
Rhodamine Phalloidin	Invitrogen	R415	1:20

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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