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Sample Preparation of Human Eye Anterior Segment for sc/snRNA-seq

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ABSTRACT

This protocol is used for sample preparation of human donor eye for single cell/single nuclei RNA sequencing. The protocol consists of dissection, fresh and frozen tissue dissociation. The dissection of both anterior segment and posterior segment are included, but only anterior segment dissociation is described in this protocol. For posterior segment dissociation, please refer to another protocol from Dr. Rui Chen lab. https://www.protocols.io/view/nuclei-isolation-for-snrna-seq-and-snatac-seq-from-bftmjnk6

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KEYWORDS

eye dissection, eye dissociation, single cell sequencing

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Dissection of Human Donor Eye

1 Recovery Conditions

For a good quality of retina and RPE, eyes should be recovered no more than 6 hours post-mortem. If more than 6 hours but less than 12 hours, eyes could still be recovered and dissected, but posterior dissection for single cell/nuclei can be omitted (part 2.3). It has been observed that the cells in the anterior segment tissue still have high viability.

2 Preparation

2.1 Prepare surgical instruments, dissection microscope, liquid nitrogen and wet ice.

2.2 For frozen tissue dissociation, prepare 20 2ml cryogenic vials labeled as optic nerve, fovea-retina, fovea-RPE, macula-retina, macula-RPE, retina-S, retina-I, retina-N, retina-T, RPE-S, RPE-I, RPE-N, RPE-T, lens, iris, ciliary, TM, cornea, limbus, sclera, followed by donor ID and collection date.
Note: S = superior, I = inferior, N = nasal, T = temporal

2.3 For fresh tissue dissociation of anterior segment, prepare seven 2ml Eppendorf tubes labeled with iris, ciliary, TM, cornea, limbus, sclera, and lens. Add 1 ml of MACS® Tissue Storage Solution (Miltenyi Biotec #**130-100-008**) in each tube on ice.

3 Dissection

3.1 Place the eye in a petri dish with PBS. Remove any orbital fat from the exterior. Cut the optic nerve and put into cryogenic vial labeled as “optic nerve” and put into liquid nitrogen.
Note: extraocular muscles and adipose can also be collected if needed.

3.2 Use a 15-degree stab knife or a surgical blade to make an incision on the sclera 5 mm (adult eye) away from the limbus. Use the dissection scissors to cut around and separate the entire anterior segment. Put the anterior segment in a separate petri dish with PBS for later dissection.

3.3 Find the optic nerve and fovea from the remaining eye cup. Cut the posterior

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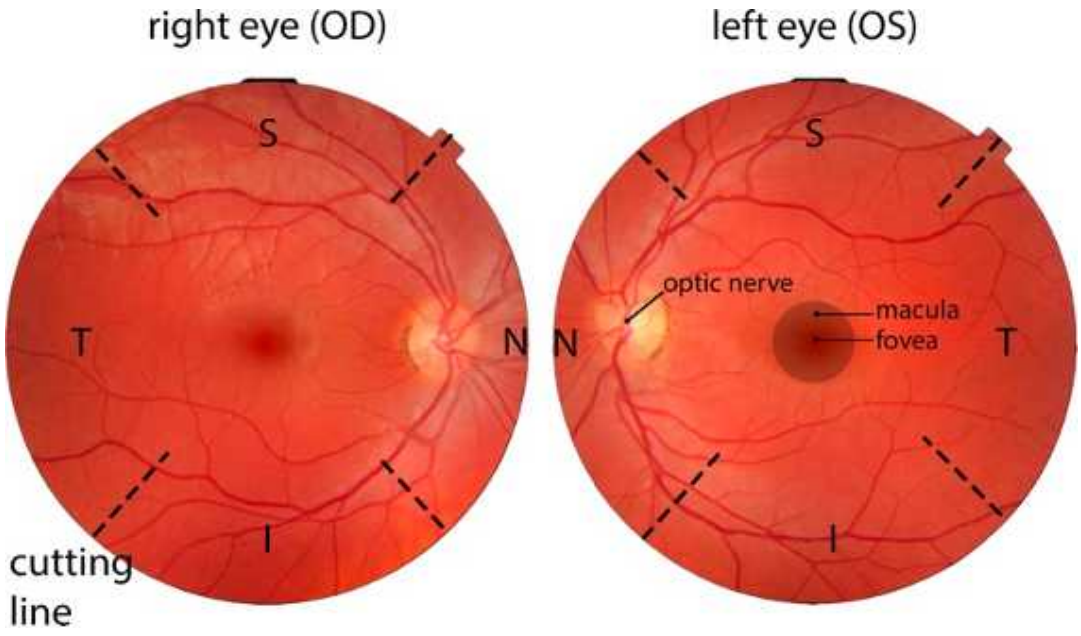
segment, including retina, RPE/choroid, and sclera, into 4 lobes. Avoid touching the posterior pole. The 4 lobes should represent the superior, inferior, nasal and temporal part of the posterior segment.



3.4 Carefully remove the vitreous body using scissors as clean as possible (this is important especially for the single cell RNA-seq).

3.5 Use a 2 mm punch to collect the fovea, place the fovea retina and fovea RPE in the corresponding cryogenic vials.
Note: from this step, snap freeze the cryogenic vials in liquid nitrogen right after each step.

3.6 Use a 6 mm punch to collect the macula, place the macular retina and macular RPE in the corresponding cryogenic vials.

3.7 Use scissors and forceps to separate the retina and RPE of each lobe (S, I, N, T) and place into each cryogenic vials.

3.8 Use a 2 mm punch to collect the optic nerve head.

3.9 Under a dissection microscope, gently use curved scissors or forceps tips to separate the lens from its suspensory ligament.

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3.10 For lens of adult donor, peel off lens capsule using two fine forceps and collect in the corresponding tube. Discard the remaining lens core. For a pseudophakic eye (intraocular lens, IOL), remove the IOL and collect the rest lens capsule. For immature lens, collect the whole lens without peeling off the capsule.

3.11 Use scissors to cut around the junction between iris and ciliary body and collect the iris. Peel off and collect the ciliary body.

3.12 Use fine forceps to grab the TM at the base of the cornea. Carefully separate the TM and collect it.

3.13 Use 4mm punch to collect the cornea, limbus and sclera.

3.14 For frozen samples, transfer the vials to -80°C freezer for short term storage (< 1 month), or to liquid nitrogen tank for long term storage. For fresh samples, proceed to tissue dissociation right away or leave overnight in tissue storage solution in 4°C.

Dissociation of Human Anterior Segment of Eye

4 Fresh tissue dissociation for scRNA-seq

4.1 Prepare Collagenase A (Sigma # 10103586001) working solution as shown in the following able by diluting the 100 mg/ml stock in HBSS.

4.2 Cut the tissue into small pieces.

4.3 Incubate the samples in Collagenase A working solution in 2 ml tubes at 37°C on a shaker.

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A	B	C	D
Tissue	Size§	Conc. of Collagenase A¶	Time of incubation†
Cornea	4mm punch X 2	1.5 mg/ml	~ 2 h
Limbus	4mm punch X 2	2 mg/ml	~ 2 h
Sclera	Bigger the better	2.5 mg/ml	> 2 h
Ciliary body	1/8 – 1/4 of all	1 mg/ml	30 min ~ 1 h
Iris	1/4 - 1/2 of all	1 mg/ml	< 30 min
TM	All	1.5 mg/ml	~1 h
Lens capsule	1/2 - all	1 mg/ml	< 30 min

§ Usually 50,000 to 200,000 cells can be obtained. Smaller tissue can be used if needed.
¶ Additional 0.5% Collagenase type 1 can also be added to limbus and sclera to accelerate the dissociation.
† Triturate the tissues using a wide bore tip every 10 – 30 min remarkably accelerates the dissociation.

4.4 Keep triturating until most of the tissues have disappeared.

4.5 Centrifuge the tubes at 500 x g for 5 min.
Note: Collagenase activity can be inhibited by lowering the temperature. No protease inhibitor is needed.

4.6 For cornea, limbus, ciliary body, and iris, remove the supernatant and re-suspend the cells in 1 ml of 0.25% Tryspin-EDTA (Gibco™ # 25200056). For sclera, TM, and lens capsule, go to step 4.9.

4.7 Incubate at 37 °C on a shaker. Triturate every 10 min.

Tissue	Time of incubation
Cornea	~ 1 h
Limbus	30 min - 1 h
Ciliary body	~ 30 min
Iris	~ 30 min
Lens	< 30 min

4.8 Check the cells every 10 min until all the cells are in single-cell form.

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4.9 Add 80 KU/mL DNase I and incubate in the current solution for another 10 min.

4.10 Transfer the cells into a 15 ml tube, add DMEM/5%FBS to 10 ml. Gently rotate the tube to mix.

4.11 Filter the cells through a pre-wet 30 µm strainer into another 15 ml tube.

4.12 Spin down the cells at 500 x g for 8 min.

4.13 Remove the supernatant and resuspend cells in appropriate amount of DMEM. Count the cell number and check the cell viability. The cells are ready for 10X.

4.14 Optional:

- Ciliary body and iris are highly pigmented tissues. Melanin pigments can sometimes hamper cell count and viability check but may not influence barcoding and sequencing according to our experience. Density gradient centrifugation can be applied to remove excessive pigments if desired. However, there is risk of losing some of the melanocytes by doing so.

1. Resuspend ciliary body and iris in 1 ml DMEM.
2. Prepare 40% OptiPrep by mixing 2 vol of OptiPrep and 1 vol of DMEM (serum free).
3. Prepare 1 ml of 30% OptiPrep by mixing 750 µl 40% OptiPrep and 150 µl DMEM.
4. In a 5 ml Falcon tube, add 1 ml of 40% OptiPrep, followed by 1 ml of 30% OptiPrep, followed by 1 ml of sample. Carefully overlay the solutions.
5. Set brake at 0. Spin down at 600 g for 15 min.
6. Most cells are at the first interface (above 30%), and the pigments are at the bottom of the tube. Collect the whole 30% and upper 1/3 of the 40% in a new 15 ml tube.
7. Add DMEM to 12 ml.
8. Spin down at 1000 g for 10 min. Set brake back to 9.
9. Remove the supernatant and resuspend cells in appropriate amount of DMEM.

5 Frozen tissue dissociation for snRNA-seq

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Note: The nuclei isolation kit (10X Genomics # PN-1000494) is suitable for less fibrous tissues (ciliary body, iris, and lens capsule), but not suitable for highly fibrous tissues (cornea, limbus, sclera, and TM)

5.1 Pre-cool all the consumables and instruments on ice.

5.2 Prepare all the required buffers as indicated in the document #CG000505 from 10X Genomics website.

5.3 Use ~10 mg of ciliary body or iris for nuclei isolation. For lens capsule, put the whole lens capsule in the dissociation tube, add 200 µl of lysis buffer and go to step 5.6.

5.4 Cut ciliary body or iris into small pieces on dry ice on a glass petri dish.

5.5 Put the petri dish on wet ice, add 200 µl of lysis buffer to the tissues, and transfer the tissues to the dissociation tube. Cut tip if needed.

5.6 Dissociate the tissues using the pestle included in the kit. Keep dissociating till the lysate is homogeneous. The ciliary body and the iris lysate should look nearly black.

5.7 Follow the manufacture's protocol for the following steps. In the final nuclei suspension, there will be melanin pigments adhere to the nuclei, and it will hamper nuclei quality check under light microscope. fluorescent dyes can be used to check the nuclei quality instead.

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