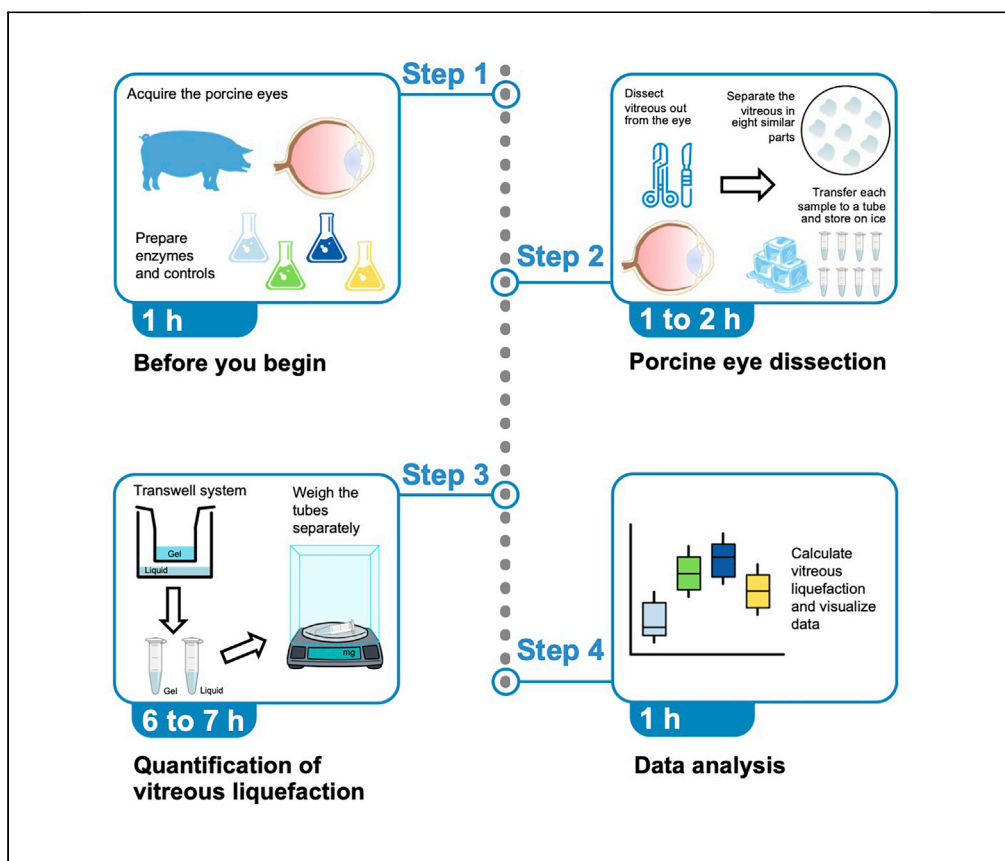


## Protocol

# Protocol to quantify enzymatic effects on vitreous liquefaction in porcine eyes using a transwell-plate system



Julian Wolf, Luís E. Sabage, Young Joo Sun, Vinit B. Mahajan

vinit.mahajan@stanford.edu

### Highlights

Transwell-plate-based protocol for quantifying vitreous liquefaction *ex vivo*

Perform vitreous separation through a process of induced syneresis

Perform multiple experiments from a porcine eye with minimal sample loss

Experimental design allows end-product analysis to account for sample loss

This protocol describes an *ex vivo* model to quantify enzymatic effects on vitreous liquefaction using porcine eyes in a transwell-plate system via induced syneresis. It provides a standardized dissection process and performs critical steps for gel-liquid separation with high precision, minimal tissue loss, and scalability. The protocol can be applied to other studies investigating vitreous liquefaction or gelatinous tissue analysis and can also serve to study vitreous liquefaction *in vivo* as it may occur during aging or disease progression.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Protocol

## Protocol to quantify enzymatic effects on vitreous liquefaction in porcine eyes using a transwell-plate system

Julian Wolf,<sup>1,4</sup> Luís E. Sabage,<sup>1,2</sup> Young Joo Sun,<sup>1</sup> and Vinit B. Mahajan<sup>1,3,5,\*</sup><sup>1</sup>Molecular Surgery Laboratory, Byers Eye Institute, Department of Ophthalmology, Stanford University, Palo Alto, CA 94304, USA<sup>2</sup>Medical Course, Bauru School of Dentistry, University of São Paulo, Bauru, São Paulo 17012-901, Brazil<sup>3</sup>Veterans Affairs Palo Alto Health Care System, Palo Alto, CA 94304, USA<sup>4</sup>Technical contact: [julian.wolf@stanford.edu](mailto:julian.wolf@stanford.edu)<sup>5</sup>Lead contact\*Correspondence: [vinit.mahajan@stanford.edu](mailto:vinit.mahajan@stanford.edu)  
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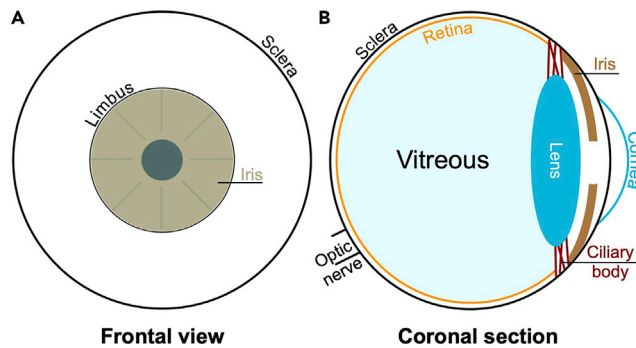
## SUMMARY

This protocol describes an *ex vivo* model to quantify enzymatic effects on vitreous liquefaction using porcine eyes in a transwell-plate system via induced syneresis. It provides a standardized dissection process and performs critical steps for gel-liquid separation with high precision, minimal tissue loss, and scalability. The protocol can be applied to other studies investigating vitreous liquefaction or gelatinous tissue analysis and can also serve to study vitreous liquefaction *in vivo* as it may occur during aging or disease progression.

## BEFORE YOU BEGIN

The human eye is filled with a gelatinous vitreous body composed mostly of water and shaped by collagen and proteoglycans (e.g., hyaluronic acid). Normal age-related processes lead to vitreous liquification when molecules bound to water are disrupted (Sebag, 1987a). This protocol describes an *ex vivo* model based on a transwell-plate system to evaluate enzyme effects on vitreous liquefaction using postmortem porcine eyes. Previous studies have investigated vitreous liquefaction by separating the gel from the liquid using a sieve, filter paper, or tweezers (Bishop et al., 1999; Brown et al., 1996; Chattopadhyay et al., 1992; Huang et al., 2018). These separation methods are imprecise, can destroy the vitreous structure, and lead to significant sample loss. Thus, it is impossible to separately calculate the weight of the gel and the vitreous, leading to misinterpretation of results (Bishop et al., 1999; Brown et al., 1996; Chattopadhyay et al., 1992; Huang et al., 2018). The process of induced syneresis (gel-liquid separation) using a transwell-plate system overcomes these challenges. Large-scale separation is performed with high precision and minimizes total sample loss because up to eight vitreous samples can be analyzed from one porcine eye. This study used postmortem porcine vitreous because of its similarity to human vitreous and its large-scale availability (Noulas et al., 2004). In addition, the protocol can also be used to study vitreous liquefaction *in vivo*, such as during aging, disease, or in animal models, as well as to analyze liquefaction of other mammalian vitreous samples or other gelatinous tissues and tissues with a large extracellular matrix composition (e.g., lens, fat, and cartilage) (Labiris et al., 2016; Shen et al., 2019). Before implementing this protocol, researchers should become familiar with eye anatomy, the structural composition of the vitreous, and handling of postmortem animal specimens. In addition, the different conditions and enzymes that will be tested and the number of eyes needed should be determined in advance. The described protocol, for example, was developed to test three enzymes and a no-enzyme control with ten biological replicates each ( $n = 10$  per each group), requiring 40 vitreous samples obtained





**Figure 1. Anatomy of the eye**

(A) Frontal view of the eye. During dissection, the incision should be performed in the red dotted line.  
(B) Coronal cut of eye showing the structures that surround the vitreous.

from five porcine eyes. Users should also familiarize themselves with mechanistic principles of vitreous liquefaction and associated enzymes (Huang et al., 2018), which can influence the experimental design, outcomes, and interpretation.

### Anatomy of the eye and structural compounds of the vitreous

The porcine globe is slightly smaller (average 20 × 24 × 25 mm) than the human eye (average 24 × 24 × 24 mm; length × width × height) (Augusteyn et al., 2012; Middleton, 2010). Key anatomical landmarks researchers must know to conduct the protocol are the cornea, which is the transparent window at the front of the eye, and the limbus, which is the border between the cornea and the non-transparent sclera (Figure 1A). The vitreous is a transparent gelatinous fluid that fills the posterior chamber of the eye and is surrounded by the retina, ciliary body, and lens (Figure 1B) (Middleton, 2010; Sebag, 1992). It is composed of about 98% water molecules, which are bound to glycosaminoglycans and parallel collagen fibers that extend into the superficial layers of the retina (Sebag, 1992). The structure and function of the vitreous is crucial to vision. Both inherited and acquired eye diseases (e.g., posterior vitreous detachment or retinal detachment, and Marfan's syndrome) (Holekamp, 2010), as well as aging, cause the vitreous to undergo structural changes that lead to liquefaction (le Goff and Bishop, 2008; Sebag, 1987b). These processes are thought to be the continuous reorganization of hyaluronic acid and collagen networks (Kaprinis et al., 2016; Sebag, 1987a, 1987b). Understanding the enzymes involved in the process of liquefaction is key to mapping molecular targets for new intraocular drug development. The aim is to find efficient, less invasive treatments for vitreoretinal diseases that improve patients' visual outcomes and decrease surgical complication rates (e.g., endophthalmitis, cataract, and glaucoma) (Yau et al., 2018).

### Acquisition and handling of postmortem porcine eyes

Porcine eyes can be acquired at a local slaughterhouse. The eyes should be transported in DPBS in a waterproof bag, which is kept on ice or a cooling aggregate in a closed temperature isolating box at 4°C. It is recommended to process the eyeballs immediately. If an immediate dissection is not possible, it should be performed within 24 h postmortem to avoid substantial alterations of biomolecules in the vitreous. Before the experiment, cleaning with DPBS (Dulbecco's Phosphate Buffered Saline) is essential to remove blood and other fluid from the ocular surface. Eyes with physical damage or high contamination that cannot be removed by antiseptics, such as povidone-iodine, and washing with DPBS should not be used.

From each eye, it is possible to acquire approximately 3,500 μL of vitreous (standard deviation is approximately 400 μL). After dividing the vitreous into individual samples, we found that the sum of the volumes of the individual samples is about 15% smaller than the total volume of one vitreous. The estimated volume per sample can be calculated using the following formula:

**Table 1. Enzymes used in the experiment**

Enzyme	Concentration in DPBS	Volume per sample
Collagenase from <i>Clostridium histolyticum</i>	0.5 mg/mL	100 $\mu$ L
Trypsin from porcine pancreas	2.0 mg/mL	100 $\mu$ L
Hyaluronidase from bovine testes	1.0 mg/mL	100 $\mu$ L

$$\text{estimated volume per sample } (\mu\text{L}) = \frac{(3,500 \mu\text{L})}{\text{number of samples per eye}} * 0.85$$

With eight samples per eye, around 370  $\mu$ L per sample can be expected (standard deviation is approximately 65  $\mu$ L). Notice that this volume will vary and will only match roughly the desired volume.

The following formula can be used to calculate how many eyes the experiment will require:

$$\text{eyes} = \frac{(\text{replicates per condition}) \times (\text{number of enzymes} + 1)}{(\text{number of samples per eye})}$$

In this protocol, we collected eight vitreous samples from each of five porcine eyes. This allowed for ten biological replicates per condition using three enzymes and a negative control. Generally, we recommend preparing more samples than needed. Considering the example mentioned above, six eyeballs should be prepared.

### Vitreous liquifying enzyme preparation

⌚ Timing: 1 h

The enzymes used in the experiment—collagenase, trypsin, and hyaluronidase—were previously described to selectively digest specific vitreous structural compounds and change its mechanical properties (Huang et al., 2018). Start preparing the enzymes approximately 1 h prior to the eye dissection.

1. Prepare 100  $\mu$ L of DPBS per sample; follow Table 1 for enzyme preparation.

**Note:** Prepare additional volume (about 10%) to account for potential loss from pipetting.

**⚠ CRITICAL:** Store the prepared enzymes on ice (4°C) until use to minimize enzyme-activity loss.

2. Per condition, prepare 3 microcentrifuge tubes for the whole sample, separated gel and separated fluid collection.

**⚠ CRITICAL:** Precise labeling of the tubes is essential. Distribute the samples from one eye across all experimental conditions to account for potential variability between different eyes.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Biological samples</b>		
Fresh porcine eyes (transport on ice, dissect within 24 h after death)	Local slaughterhouse	N/A
<b>Chemicals, peptides, and recombinant proteins</b>		
Collagenase from <i>Clostridium histolyticum</i>	MilliporeSigma, HE, Germany	Cat#C1764
DPBS, no calcium, no magnesium	Thermo Scientific, MA, USA	Cat#14190136

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hyaluronidase from bovine testes	MilliporeSigma, HE, Germany	Cat#H3506
Povidone-iodine (Betadine® Solution)	Avrio Health, CT, USA	67618-150-08
Trypsin from porcine pancreas	MilliporeSigma, HE, Germany	Cat#T7409
<b>Software and algorithms</b>		
R Statistical Software	R Foundation, Vienna, Austria	<a href="https://www.r-project.org/">https://www.r-project.org/</a>
<b>Other</b>		
Allegra X-14R Benchtop Centrifuge	Beckman Coulter, IN, USA	Cat#A99465
Conical centrifuge tubes – 50 mL	Corning™ Falcon™	Cat#352070
Corning® Transwellreg; 12 mm, 0.4 μm pore polycarbonate membrane	Corning, NY, USA	SKU: CLS3401-48EA
Dissecting scissor	MilliporeSigma, HE, Germany	SKU: Z265977-1EA
Dressing tissue forceps	MilliporeSigma, HE, Germany	SKU: F3892-1EA
Incubator	BINDER, MD, USA	Cat#08-111-399
Microcentrifuge tubes – 1.5 mL	Thermo Scientific, MA, USA	Cat#3451
Micro-dissecting forceps	MilliporeSigma, HE, Germany	SKU: F4142-1EA
Petri dish plate	Corning, NY, USA	REF: 351029
Pipette 20–200 μL	Eppendorf, HH, Germany	SKU: 3123000055
Pipette 100–1,000 μL	Eppendorf, HH, Germany	SKU: 3123000063
Precision balance	METTLER TOLEDO, OH, USA	Material#: 30315631
Razor blade	STANLEY, CT, USA	Cat#11-515
Scalpel blade #11	iMed Scientific, VA, USA	SKU: IMS-CBLD11
Table Minicentrifuge	Waverly, IA, USA	SKU: C100

## MATERIALS AND EQUIPMENT

### Enzymes for vitreous liquefaction

#### Collagenase

Reagent	Final concentration	Amount
Collagenase from <i>Clostridium histolyticum</i>	0.5 mg/mL	0.05 mg
DPBS	N/A	100 μL

**Note:** The amount described is for one sample. Multiply the amounts for the number of replicates plus 10% for potential loss from pipetting.

#### Trypsin

Reagent	Final concentration	Amount
Trypsin from porcine pancreas	2 mg/mL	0.2 mg
DPBS	N/A	100 μL

**Note:** The amount described is for one sample. Multiply the amounts for the number of replicates plus 10% for potential loss from pipetting.

#### Hyaluronidase

Reagent	Final concentration	Amount
Hyaluronidase from bovine testes	1 mg/mL	0.1 mg
DPBS	N/A	100 μL

**Note:** The amount described is for one sample. Multiply the amounts for the number of replicates plus 10% for potential loss from pipetting.

### STEP-BY-STEP METHOD DETAILS

This section provides detailed steps to acquire porcine vitreous samples and analyze alterations in the gel-liquid ratio after enzyme activity. The dissection is performed to maintain the vitreous structure without other attached tissues (e.g., iris or retina), thus reducing sample contamination and enzyme cross-effect. A transwell-plate system is used to separate the liquid from the gel, minimizing sample loss to obtain accurate measurements of liquefaction changes.

#### Porcine eye dissection

⌚ Timing: 1–2 h

The described protocol is key to obtaining viable vitreous samples with proper scalability and reaching eight samples with approximately 370  $\mu\text{L}$  each (standard deviation about 65  $\mu\text{L}$ ). Keep the porcine eyes that are not being dissected at 4°C (or on ice) to minimize tissue damage. Prepare and organize the surgical tools prior to the procedure (Figure 2A). For self-protection, the researcher should follow the following safety recommendations. Use appropriate personal protective equipment, such as gloves and a closed lab coat during the experiment. To minimize exposure to potential biological hazards, the ocular surface should be decontaminated with antiseptics, such as povidone-iodine, followed by washing with DPBS. In addition, we highlight the step of the first incision using the scalpel because there is high risk for injury if the eyeball is not held securely. The steps of the dissection are demonstrated in Methods video S1.

1. To decontaminate the ocular surface:
  - a. Place the porcine eye in a 50 mL falcon tube containing povidone-iodine for 3 min.
  - b. Transfer it to a tube with DPBS to wash off remaining antiseptics.
  - c. Transfer the porcine eye to a 100 mm  $\times$  15 mm petri dish plate.
  - d. With clean forceps, hold the eye at the sclera or adjacent conjunctival tissue, and wash the eye from the cornea towards the posterior part of the globe 3 times with 1,000  $\mu\text{L}$  DPBS using a 1,000  $\mu\text{L}$  pipette.

**Note:** This step removes remaining external contaminants (e.g., dust, dirt, or non-ocular tissue debris from enucleation).

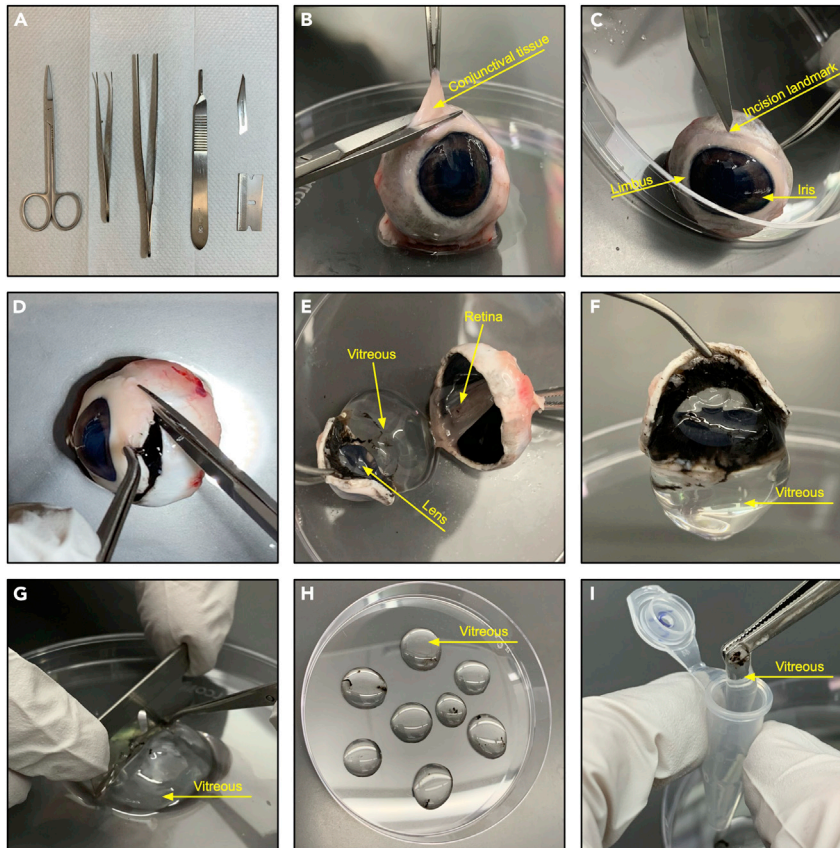
2. Transfer the eye to a fresh plate. Using scissors, remove conjunctival and muscle tissue in the area between the limbus and 5–6 mm posterior to the limbus to facilitate access to the sclera (Figure 2B).

**Note:** Avoid damaging the sclera while cutting the conjunctiva.

3. Using a clean surgical scalpel with a new, sharp blade (size 11), make the first incision 3–4 mm posterior of the limbus (Figures 2C and 3).

**Note:** Use the wall of the dish and a tweezer to stabilize the eyeball. Make sure that the force you apply with the scalpel points to the center of the eye to prevent it from rotating (Figure 2C).

**⚠ CRITICAL:** The sclera is thick and rigid, making it hard to penetrate with the scalpel blade. There is a high chance for injury if the eyeball is not held securely.



**Figure 2. Dissection of vitreous samples from porcine eyes**

(A) Instruments needed for dissection.

(B) Remove remaining conjunctival and muscle tissue in the area between the limbus and 5–6 mm posterior to the limbus.

(C) Make the first incision 3–4 mm posterior to the limbus using a surgical scalpel with blade size 11. Use the wall of the dish and a tweezer to stabilize the eyeball and make sure that the force you apply with the scalpel points to the center of the eye to prevent it from rotating.

(D) Insert a scissor into the incision and cut 360 degrees at 3–4 mm parallel to the limbus to separate the anterior from the posterior part of the eye. Use a tweezer to hold the opened edge of the anterior segment to facilitate separation.

(E) Next, hold the anterior and posterior parts with one tweezer each and gently pull them apart. During this process, the vitreous should adhere to the anterior part and detach from the retina.

(F) Hold up the anterior part with the vitreous using a tweezer and use a second tweezer to carefully separate the vitreous from the structures of the anterior segment. The iris, ciliary body and lens should remain with the anterior segment.

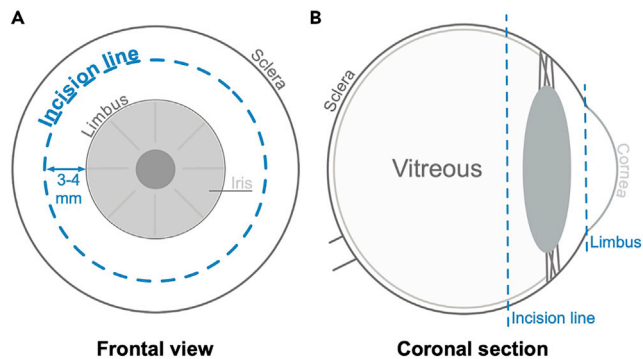
(G and H) Cut the vitreous in eight equally sized parts using a razor blade. If this does not completely divide the vitreous, a small tweezer can be used to carefully move the vitreous relative to the blade.

(I) Transfer each vitreous sample in a labeled tube using a tweezer. Store the samples on ice.

**△ CRITICAL:** The location of the first incision site is critical for the clean separation of the vitreous from the other ocular tissues. The location closer to the limbus may result in the vitreous staying intact with the posterior segments ([troubleshooting 1](#)). The location farther from the limbus has a higher chance of fragmenting the vitreous tissues into small pieces.

4. Insert scissors into the incision and cut 360 degrees in the sclera at 3–4 mm parallel to the limbus (see blue dotted line in [Figure 3](#)) to separate the anterior from the posterior segment of the eye.

**Note:** Use forceps to hold the opened edge of the anterior segment to facilitate separation.



**Figure 3. Performing the incision in the porcine eye**

(A) The incision must be performed between 3 to 4 mm from the limbus. Frontal view of the eye.  
(B) Coronal section of the eye.

**Note:** Always having one of the instruments inside the eye can help to facilitate dissection; when the tweezer is not holding the sclera, the tip of the scissors should keep the incision open (Figure 2D).

5. Hold each anterior and posterior part with forceps and gently pull them apart. During this process, the vitreous should adhere to the anterior part and detach from the retina (Figure 2E).

**Note:** Retina tissue detached from the posterior segment may cover the vitreous tissues (troubleshooting 2). Gently remove the attached retinal tissue to minimize contamination.

6. Use forceps to lift and hold the anterior segment with the vitreous, and use a second pair of tweezers to carefully separate the vitreous from the structures of the anterior segment.

**Note:** Rotating the anterior segment and utilizing natural gravity pull-down can facilitate the separation (Figure 2F).

**Note:** The iris, ciliary body and lens should remain with the anterior segment with a proper incision made in steps 3 and 4. However, it is possible that fragmented iris tissue may follow the vitreous tissue (troubleshooting 3).

7. With a razor blade, cut the vitreous into four to eight equally sized parts.

**Note:** If this does not completely divide the vitreous, small forceps can be used to carefully move the vitreous relative to the blade (Figures 2G and 2H).

8. Transfer each vitreous sample to the labeled 1.5 mL microcentrifuge tube using forceps.

**△ CRITICAL:** Use tubes from the same batch to avoid large weight differences in the next steps (Figure 2I).

9. Store the tubes on ice (4°C).
10. Repeat the steps to acquire the amount of sample required for the experiment.

### Quantification of vitreous liquefaction

⌚ Timing: 6–7 h



This part of the protocol describes the process of enzyme incubation and gel-liquid separation. A transwell-plate based system was used to induce vitreous syneresis and precisely measure the relative enzymatic changes in the gel and liquid with scalability and minimal sample loss. Note that the inter-pig eye variability in control samples is slightly higher than the intra-pig eye variability: the standard deviation of each comparison group was  $\sim 6.9\%$  and  $\sim 1.5\%$ , respectively. Therefore, we recommend distributing the samples from one eye across all experimental conditions to minimize overall variability and using at least 10 replicates per condition. Both inter- and intra-pig eye variability may increase in enzyme-treated testing groups compared to control groups. For example, our collagenase-treated samples had standard deviation of  $\sim 13.9\%$  for inter-pig eye comparisons and  $\sim 8.1\%$  deviation for intra-pig eye comparisons. These changes in variability may depend on specific assay conditions and enzymes being tested, thus we recommend investigating the inter- and intra-pig eye variabilities for each different enzyme before interpreting the results.

11. With a pipette, inject 100  $\mu\text{L}$  of enzyme solution in the center of each vitreous sample in the microcentrifuge tube.

**Note:** We used each following enzymes in ten replicates: collagenase (0.5 mg/mL), trypsin (2.0 mg/mL), hyaluronidase (1.0 mg/mL), or your target enzyme. Use DPBS as a control.

**Note:** Refer to the manufacturer's instructions for optimal and maximum storage conditions of each enzyme. As suggested by the manufacture, the enzymes used in this protocol should be stored under the following conditions: collagenase:  $-20^{\circ}\text{C}$  for up to one year, trypsin  $-20^{\circ}\text{C}$  for up to one year, hyaluronidase:  $-20^{\circ}\text{C}$  (maximum storage time unspecified).

12. Incubate the samples in closed microcentrifuge tubes at  $37^{\circ}\text{C}$  for 3 h.

**Note:**  $37^{\circ}\text{C}$  is the optimal temperature for the selected enzymes' activity (information provided in the products' description).

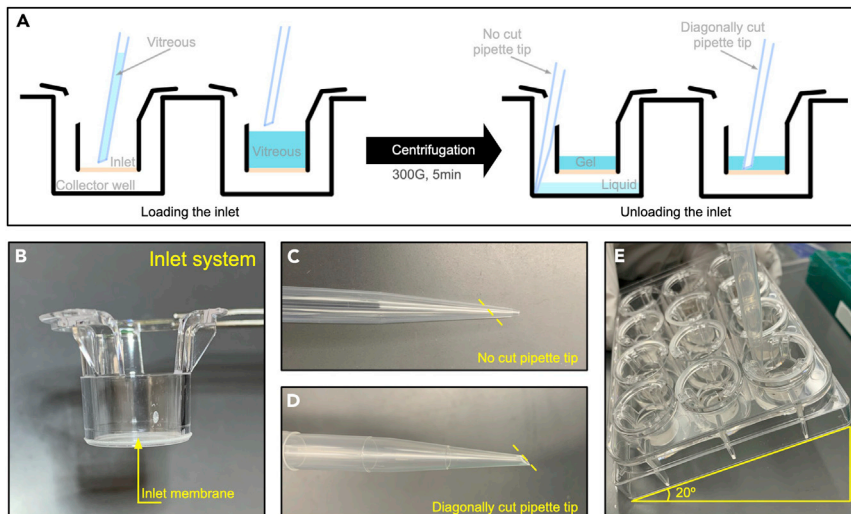
13. Weigh each sample in the tube using a laboratory milligram scale with a windshield.
14. Briefly centrifuge the tubes for 1–2 s using the table minicentrifuge to precipitate condensed fluid from the top of the tubes.
15. In case you use more than one transwell plate, label the plate with a number and prepare a table indicating which enzyme is in each well.
16. Transfer the samples from the tube to the inlet (apical side) of the transwell using a 1,000  $\mu\text{L}$  pipette (Figures 4A and 4B).

**Note:** Cut 3 mm of the tip of the pipette diagonally to increase tip diameter and allow easier transfer of the gel (Figures 4C and 4D).

**Alternatives:** Centrifugation filter tubes (e.g., Corning centrifuge tube filters #8163) might also be used for gel-liquid separation (comparable pore size and membrane material). However, the maximum volume which can be transferred to the centrifugation tubes is 500  $\mu\text{L}$ . Since some samples (+100  $\mu\text{L}$  enzyme) will have a larger volume (with 8 samples per eye), the tubes can only be an alternative for samples with a total volume below 500  $\mu\text{L}$ . In addition, the centrifugation parameter may need to be modified.

17. Briefly centrifuge the tubes using the benchtop centrifuge and use a 200  $\mu\text{L}$  pipette tip to transfer the residual sample to the inlet.

**Note:** The residual fluid has a low viscosity and can be transferred easily using the 200  $\mu\text{L}$  pipette tip.



**Figure 4. Using the transwell-plate system**

(A) Upload the vitreous sample in the inlet and centrifuge the transwell. Insert the pipette tip in one of lateral openings of the inlet to reach the collection well and remove the fluid part. When there is no more fluid in the collection well, remove the gel part using a cut pipette tip.

(B) Empty inlet.

(C) Standard pipette tip.

(D) Pipette tip diagonally cut for gel transference.

(E) Bend the transwell system and remove the liquid part in the collection system using one of the holes in the inlet.

**△ CRITICAL:** To reduce the total loss in the system, it is important to transfer as much fluid as possible from the tube to the inlet.

18. Centrifuge the transwell plate at  $300 \times g$  for 5 min.

19. Remove the fluid from the collection well using a 1,000  $\mu\text{L}$  pipette without removing the inlet from the well and transfer the fluid to a new labeled tube.

**Note:** The pipette tip can reach the collection well through one of the lateral openings in the inlet. During this process, hold the transwell plate at  $20^\circ$  to accumulate the liquid on one site of the collection well (Figure 4E).

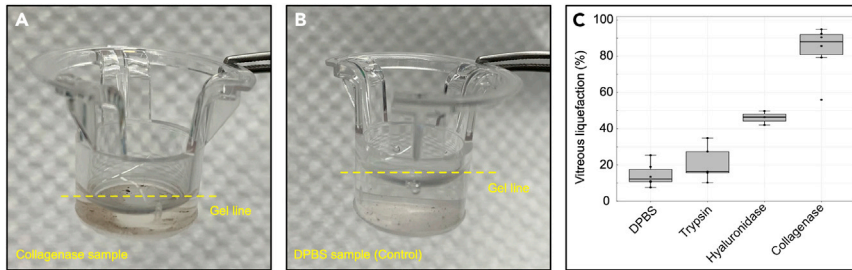
**△ CRITICAL:** To reduce the total loss in the system, transfer as much fluid as possible.

20. Centrifuge the transwell plate again at  $300 \times g$  for 5 min to separate the remaining liquid from the gel vitreous.

**Note:** Our experiments revealed that the above-mentioned centrifugation parameters ( $300 \times g$  for 5 min) demonstrated clear differences between control and enzyme treated samples without a significant disruption of the vitreous gel structure in control samples. Researchers might consider changing centrifugation parameters (e.g., other tissues or other enzymes with a weaker effect on liquefaction) (troubleshooting 4).

21. For a second time, remove the fluid from the collection well as described in step 19, and transfer it to the same labeled tube.

22. Transfer the gel from the inlets to a new labeled tube using a pipette with a diagonally cut 1,000  $\mu\text{L}$  tip (opening diameter should be around 3 mm; Figures 4A, 4C, and 4D).



**Figure 5. The transwell-plate system allows to quantify the effect of different enzymes on vitreous liquefaction *in vitro***

(A and B) Vitreous liquefaction is exemplified by a sample treated with collagenase (A) compared to a PBS control sample (B).

(C) The percent of vitreous liquefaction is visualized as a box-and-whisker plot for three different enzymes compared to PBS control samples (n = 5 for each group).

**Note:** If the vitreous cannot be sucked into the tip, try to increase the diameter of the tip's opening.

△ **CRITICAL:** The inlet membrane is fragile; do not apply force towards it, otherwise the membrane will be perforated, increasing sample loss ([troubleshooting 5](#)).

△ **CRITICAL:** It is critical that as much gel as possible is transferred from the inlet to the labeled tube.

23. Weigh the tubes to determine gel and liquid weight separately for each sample.
24. Use the sample immediately for analysis (e.g., analysis of cleavage products, or structural analysis).

**Optional:** If the same vitreous is being used for further experiments, store the tubes at  $-80^{\circ}\text{C}$ .

## EXPECTED OUTCOMES

The results of this protocol are expected to precisely quantify and compare vitreous liquefaction caused by different enzymatic activity in the vitreous sample, an example is shown in [Figure 5](#). In addition, the protocol can be used to screen the effect of biochemical manipulation on vitreous liquefaction with high throughput. In accordance with other studies ([Huang et al., 2018](#)), it is expected that collagenase, trypsin, and hyaluronidase cause vitreous liquefaction of different degrees compared to DPBS treated samples. The large range of vitreous liquification from near complete liquefaction by collagenase and minimal liquefaction in control samples indicates that weaker effects of other enzymes are likely to be detected as well. Results should be interpreted according to the enzymes used, their expression in healthy or pathological vitreous, and environmental or disease triggers that could alter enzyme activity.

## QUANTIFICATION AND STATISTICAL ANALYSIS

To interpret the data, calculate the mean and standard deviation of the loss with the formula:

$$\text{sample loss} = \text{initial weight} - (\text{gel weight} + \text{liquid weight})$$

Samples with a loss above one standard deviation above the mean should be excluded. Subtract the loss from the initial weight to find the "final total weight" for each sample. Calculate the relative liquid and gel as follows:

$$\text{liquid \%} = \frac{\text{liquid weight} * 100}{\text{final total weight}}; \text{gel \%} = \frac{\text{gel weight} * 100}{\text{final total weight}}$$

**Table 2. Example of raw data and calculation of vitreous relative liquefaction**

Group	Total weight (mg)	Liquid weight (mg)	Gel weight (mg)	Sample loss (mg)	Final total weight (mg)	Liquid %	Gel %
Collagenase 0.5 mg/mL	545	383	100	62	483	79.3	20.7
Trypsin 2.0 mg/mL	637	464	91	82	555	83.6	16.4
Hyaluronidase 1.0 mg/mL	441	157	217	67	374	42.0	58.0
DPBS (control)	575	69	445	61	514	13.4	86.6

An example of the calculation can be seen in [Table 2](#).

## LIMITATIONS

Our method allows for testing different enzyme effects on vitreous samples through an improved dissection method and a unique syneresis process using a transwell-plate system. Similar to clinical human patient specimens, porcine eyes vary by animal (e.g., genetic background and lifestyle). This protocol allows the division of vitreous samples from the same eye to different conditions, minimizing interindividual variability. Statistical analysis with a large sample size and biological replicates might be desired to overcome this potential limitation. In parallel, acquiring the eyes from trusted slaughterhouses is desired to acquire eyes under more controlled conditions (e.g., time after enucleation, animal health, and transportation).

## TROUBLESHOOTING

### Problem 1

The vitreous stays attached to the posterior part of the eye ([step-by-step method details](#): porcine eye dissection; step 3).

### Potential solution

A potential reason for the attachment is that the incision in the sclera is too close to the limbus so that the pars plana stays with the posterior part of the eye, leaving the vitreous with fewer attachments to the anterior segment. When pulling both halves apart, the vitreous will remain attached to the posterior segment, thus it will not be possible to freely separate them without destroying the vitreous' structure ([Figure 6A](#) and [Methods video S2](#)). If the incision is too close, repair it by expanding it posteriorly with scissors, maintaining the distance of 3–4 mm posterior to the limbus while cutting.

### Problem 2

Retinal tissues are still attached to the vitreous after separating the posterior and anterior halves of the eye ([step-by-step method details](#): porcine eye dissection; step 5).

### Potential solution

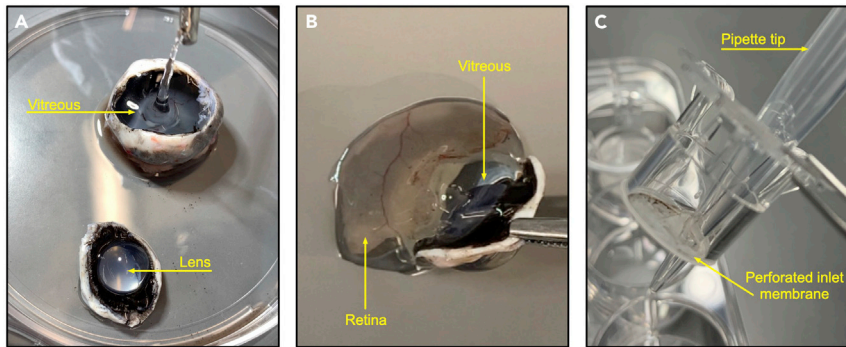
Hold the eye with tweezers at the sclera of the anterior segment and, with another blunt closed tweezer, gently remove the retinal tissue from the vitreous. Do not try to grab the retina or the vitreous to remove the tissue, which may destroy the vitreous' structure ([Figure 6B](#) and [Methods video S3](#)).

### Problem 3

After dissection, there are many iris and ciliary body remnants in the vitreous ([step-by-step method details](#): porcine eye dissection; step 6).

### Potential solution

A few iris or ciliary body remnants should not influence the gel-liquid separation (such as in [Methods video S1](#)). However, if the contamination is too high, it might block the inlet membrane and will not permit the transference of the liquid part of the vitreous to the bottom of the transwell, thus corrupting the liquefaction results. If there are more than a few pigmented particles in the dissected vitreous



**Figure 6. Examples of potential troubleshooting**

(A) The vitreous stays attached to the posterior part of the eye, potentially because the incision was too close to the limbus.

(B) Retina is still attached to the vitreous.

(C) Perforated membrane of the inlet.

or if a sample with much contamination turns out to be an outlier, the researcher should consider excluding the sample.

#### Problem 4

The vitreous liquefaction is higher than expected in the control group or lower than expected in the enzyme treated group ([step-by-step method details: quantification of vitreous liquefaction](#); step 20).

#### Potential solution

One solution can be the centrifugation parameter. If the liquefaction is lower than expected, the centrifugation speed or time can be increased and vice versa. Another option is to modify the enzyme dose or incubation time.

#### Problem 5

The inlet membrane breaks the transwell membrane while removing the gel from the inlet because too much strength is applied to the pipette tip ([Figure 6C](#)) ([step-by-step method details: quantification of vitreous liquefaction](#); step 22).

#### Potential solution

Be gentle while manipulating the inlet. Softly touch the membrane with the tip of the pipette. If the membrane is perforated, calculate the amount of the sample loss as described above. If the sample loss is higher than one standard deviation from the mean, exclude the sample.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vinit B. Mahajan ([vinit.mahajan@stanford.edu](mailto:vinit.mahajan@stanford.edu)).

### Materials availability

All stable reagents generated in this study are available from the [lead contact](#) with a completed Materials Transfer Agreement.

### Data and code availability

All data generated in this paper will be shared by the [lead contact](#) by request.

Any additional information required to reanalyze the data reported in this work is available from the [lead contact](#) upon request.

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101754>.

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### AUTHOR CONTRIBUTIONS

Study concept and design: J.W., L.E.S., Y.J.S., V.B.M. Acquisition of data: J.W., L.E.S. Data analysis and interpretation: J.W., L.E.S., Y.J.S., V.B.M. Drafting of the manuscript: J.W., L.E.S. Critical revision of the manuscript: Y.J.S., V.B.M. Obtained funding: V.B.M. Administrative, technical, and material support: Y.J.S., V.B.M. Study supervision: Y.J.S., V.B.M.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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