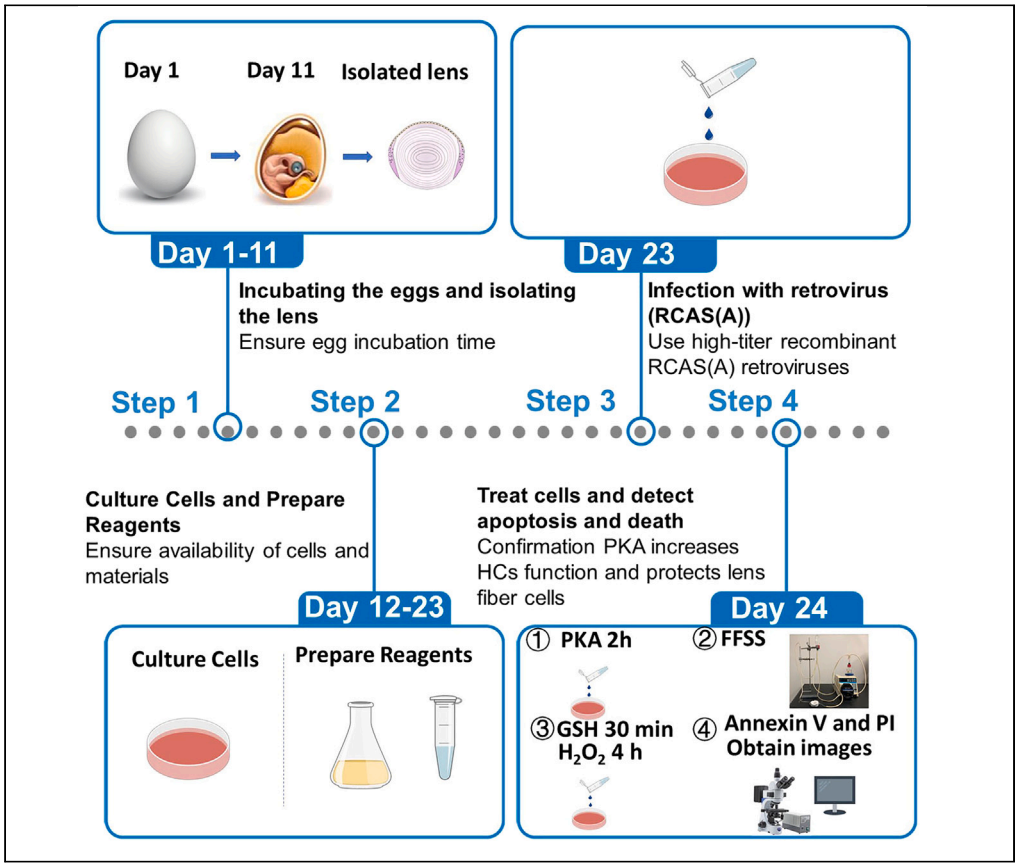
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for altering connexin hemichannel function in primary chicken lens fiber cells using high-titer retroviral RCAS(A) infection



Connexins (Cxs) play a crucial role in maintaining lens transparency. Here, we present a protocol for altering Cx hemichannel (HC) function in primary chicken lens fiber cells using high-titer retroviral RCAS(A) infection. We describe steps for incubating eggs, isolating lenses, culturing cells, preparing reagents, and infecting cells. We then detail cell treatment and detection of apoptosis and death. This protocol can assess protein kinase A, HC activity, and increased glutathione transport for protecting lens fiber cells against oxidative stress.

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

Yu Du, Francisca M. Acosta, Jianping Zhang, Yuxin Tong, Yumeng Quan, Sumin Gu, Jean X. Jiang

duyuyanke@163.com (Y.D.)
jiangj@uthscsa.edu (J.X.J.)

Highlights

Expression of exogenous connexin HCs in chicken primary retrovirus-infected lens cells

Use of FFSS to simulate shear forces generated by lens microcirculation

Study of PKA activation enhances HCs to protect lens cells from H_2O_2-induced cell death

Du et al., STAR Protocols 4, 102564
December 15, 2023
https://doi.org/10.1016/j.xpro.2023.102564

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for altering connexin hemichannel function in primary chicken lens fiber cells using high-titer retroviral RCAS(A) infection

Yu Du,[1,2,3,*] Francisca M. Acosta,[1] Jianping Zhang,[1] Yuxin Tong,[1] Yumeng Quan,[1] Sumin Gu,[1] and Jean X. Jiang[1,4,*]

[1]Department of Biochemistry and Structural Biology, University of Texas Health Science Center, San Antonio, TX 78229-3900, USA

[2]Department of Ophthalmology, Lanzhou University Second Hospital; Second Clinical School, Lanzhou University, Lanzhou, Gansu 730000, China

[3]Technical contact

[4]Lead contact

*Correspondence: duyuyanke@163.com (Y.D.), jiangj@uthscsa.edu (J.X.J.)
https://doi.org/10.1016/j.xpro.2023.102564

SUMMARY

Connexins (Cxs) play a crucial role in maintaining lens transparency. Here, we present a protocol for altering Cx hemichannel (HC) function in primary chicken lens fiber cells using high-titer retroviral replication competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor (A) infection. We describe steps for incubating eggs, isolating lenses, culturing cells, preparing reagents, and infecting cells. We then detail cell treatment and detection of apoptosis and death. This protocol can assess protein kinase A, HC activity, and increased glutathione transport for protecting lens fiber cells against oxidative stress.

For complete details on the use and execution of this protocol, please refer to Liu et al.,[1] Riquelme et al.,[2] Shi et al.,[3] Jiang,[4] and Rath et al.[5]

BEFORE YOU BEGIN

The following protocol describes the specific steps for using chicken embryos and has been reviewed and approved by our Institutional Animal Care and Use Committee (IACUC). Prior to initiating the experiment, it is essential to ensure the appropriate selection of experimental models and adequate preparation and availability of reagents. For the study of lens primary cells, the average cell apoptosis/necrosis area ratio of 5 fields of view per sample results in one experimental data point. Each experiment should be repeated at least 3 times. Protein kinase A (PKA) activation enhances the functionality of both Cx50 gap junctions (GJs) and Cx hemichannels (HCs) by stabilizing the channel in a more conductive configuration. This protocol allows for the 7 investigation of the function of HCs, and enables the detection of the apoptosis/necrosis in lens fiber cells and intracellular GSH changes caused by changes in HCs function.

Preparation of site-directed mutagenesis and high-titer recombinant RCAS(A) retrovirus

Timing: 1–7 days (depending on initialization step)

1. This step relates to the preparation of a recombinant RCAS(A), for a detailed protocol, please refer to Jiang.[4]

Pause point: After collections of high-titer retroviral stocks, next steps can be continued once ready. Keep at −80°C when not in use (≤2 months).

Preparation of primary chicken lens cells – Day 1–23

Timing: ~11 days (day 1–11; for step 2)

Timing: ~2–3 h (day 11; for step 3)

Timing: ~12 days (day 12–23; for step 4)

2. Incubate 60–90 Texas White Leghorn fertilized eggs in a 37.5°C constant temperature incubator until about day 11 (Embryo 11, E 11).

Note: After setting up the incubation process in a self-regulated temperature incubator, proceed in adding water every day to ensure sufficient humidity (~60%) to provide an optimal incubation environment.

3. Dissect lenses from 11-day-old chick embryos and extract cells.

Note: For issue with lens extraction please See troubleshooting problem 1.

Note: Before extracting primary chicken lens cells, the eggs should be sterilized with 70% ethanol.

   a. Transfer the hatched eggs from the constant temperature incubator to the ultra-clean bench, sterilize the eggshell with 70% ethanol, break the head of the eggshell, gently tear the allantoic membrane with clean tweezers to expose the chicken embryo, and finally clamp it from the amniotic cavity. Take the chicken embryos and put them in a dish. The chicken embryo cornea was torn off with micro forceps, the lens was gently clamped to prevent lens rupture and damage, and the lens was quickly transferred to a tube containing TD buffer.
   b. Wash lens with TD-buffer twice.
   c. Digest lens with 0.1% trypsin in 10 mL TD-buffer at 37°C for 20 min.
   d. Homogenize properly in complete medium for chicken lens primary cells.
   e. Count living cells and seed at the density of 4×10^5 cells/well or 2×10^6 cells/dish in 0.014% type I collagen-coated (*to facilitate adherence and growth, precoated for 1 h*) 12-well plates or 60-mm dishes, respectively in complete media.

4. Primary chicken lens cells culture.
   a. Incubate cells in a 37°C incubator supplied with 5% CO_2 and change media every two days.
      i. On day 16–17, lens epithelial cells reach confluence (~100%) and begin to differentiate 14 to form fiber-like lentoid structures.
      ii. Around day 19–20, some of the lens primary epithelial cells spread out and 16 gradually differentiate into lens primary cortical fiber cells, called "Lentoids".
      iii. Around day 22–23, lens primary cortical fibroblasts mature and can be used in subsequent experiments.

Note: For issues with the growth of the primary chicken lens cells (See troubleshooting problem 2)

Note: Typical photographs of lens fiber cells differentiation process (Figure 1).

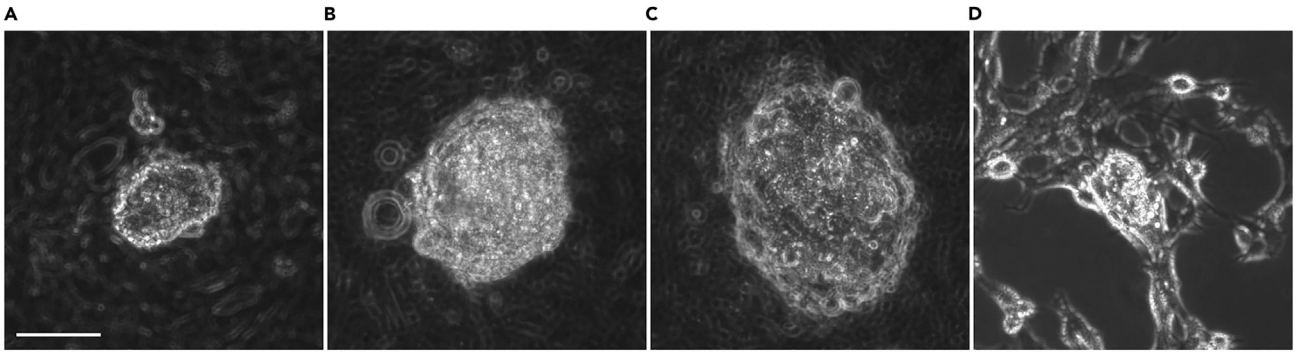


Figure 1. Successful (A, B, C) and failed (D) differentiation of lens primary cortical fiber cells
(A–D) (A) day 17, (B) day 20, (C) day 22. Scale bar = 100 μm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Recombinant RCAS(A) DNA constructs and high-titer retroviruses	Jiang and Goodenough[6]	University of Texas Health Science Center at San Antonio
Cx50H156N	Banks et al.[7]	University of Texas Health Science Center at San Antonio
Biological samples		
Fertilized white leghorn chicken eggs	Texas A&M Agriculture and Poultry Science	Fertile egg
Chemicals, peptides, and recombinant proteins		
Dimethyl sulfoxide	Thermo Fisher Scientific	Cat# MT-25950CQC
Forskolin	Thermo Fisher Scientific	Cat# 34-427-010MG
Paraformaldehyde	Thermo Fisher Scientific	Cat# 15710
Medium 199	Thermo Fisher Scientific	Cat# 11-150-059
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat# MT30001CI
Critical commercial assays		
Dead Cell Apoptosis Kit	BioLegend	Cat# 640914
Experimental models: Cell lines		
Chick lens primary cell	Jiang et al.[8]	University of Texas Health Science Center at San Antonio
Software and algorithms		
ImageJ	NIH ImageJ software	ImageJ; RRID: SCR_003070
GraphPad Prism 7 software	GraphPad software	GraphPad; RRID: SCR_000306
Other		
Microscope	Keyence (Itasca, IL, USA)	BZ-X710
FFSS FCS2/FCS3 system	Bioptechs	Cat# 12-071-20
Ova-Easy 380 Advance Series II Cabinet Incubator	Brinsea Products MJ3823C	Cat# NC1665287

MATERIALS AND EQUIPMENT

TD-buffer

Reagent	Final concentration	Amount
NaCl	140 mM	4.0908 g
KCl	5 mM	0.1864 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Na_2HPO_4	0.7 mM	0.0497 g
Glucose	5 mM	0.4504 g
Tris	25 mM	1.5143 g
Total (add ddH_2O to)	N/A	500 mL
Keep at 4°C when not in use (≤3 weeks).		

Complete medium of chicken lens primary cells		
Reagent	Final concentration	Amount
Fetal bovine serum	10%	5 mL
Penicillin-streptomycin (PS)	2%	1 mL
Medium 199	88%	44 mL
Total	**N/A**	**50 mL**
Keep at 4°C when not in use (≤2 months).		

0.014% Type I collagen solution		
Reagent	Final concentration	Amount
Type I collagen solution	0.014%	17.45 mL
glacial acetic acid solution	0.12%	0.6 mL
ddH_2O	N/A	481.95 mL
Total	**N/A**	**500 mL**
Keep at 4°C when not in use (≤3 weeks).		

FFSS loading buffer (Recording medium, RM)		
Reagent	Final concentration	Amount
NaCl	154 mM	8.99 g
KCl	5.4 mM	0.402 g
$CaCl_2$	1.8 mM	0.2 g
$MgCl_2$	1 mM	0.203 g
Glucose	5 mM	0.9 g
HEPES	10 mM	2.38 g
Total (add ddH_2O to)	N/A	1000 mL
Keep at 4°C when not in use (≤2 months).		

Note: 0.1% trypsin/TD buffer solution: add 0.1 g trypsin to 10 mL TD buffer solution, fully dissolve and mix well, then filter the solution with a 0.22 μm filter, take 1 mL of the filtrate and add it to 9 mL TD buffer solution.

Keep at 4°C when not in use (≤3 weeks).

STEP-BY-STEP METHOD DETAILS

Retrovirus RCAS(A) infection of chicken lens primary cells – Day 23

Timing: 24 h

The following experimental steps are used to infect the lens primary cells with high-titer. Recombinant replication competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor (A) (RCAS[A]) retroviruses for expression of Cx50, or impaired Cx50 HCs (Cx50 mutants H156N).

Note: For troubleshooting steps for cell infection, see troubleshooting problem 3.

1. Add 10 μL of RCAS(A) virus (~1–10 × 10^6 CFU/mL) concentrate containing vehicle, Cx50 WT, or Cx50 H156N to cultured primary chicken lens cells on 12-well plates or 60-mm dishes.
2. On the second day, replace with fresh complete medium of primary lens cells before subsequent experiments.

Note: Confirmation of cell retroviral infection can be done using western blot for anti-flag protein (Shi et al.[3]).

Note: There are no pause points from step 2 to step 11, given the nature of the experiments. The whole process needs to be done without breaks due to the time limitation of fluorescence quenching.

Pretreatment of cells with PKA activators followed by FFSS – Day 24

Timing: 8 h

The following experimental steps are used to confirm the activation of HCs in primary lens fiber cells by PKA activators. The purpose of using fluid flow shear stress (FFSS) after PKA pretreatment of primary lens fiber cells is to enhance open HCs, because in the pre-experiments, we found that the experimental results after purely using PKA to treat primary lens fiber cells were not obvious, and biologically, the lens organ is under the action of FFSS in the body.

3. Treat primary lens fiber cells with or without PKA activator.
   a. Treat primary lens fiber cells with or without PKA activator: forskolin (1 μM) for 2 h cultured in a 60 mm dish with a 40 mm cell culture slide within it.
   b. Collect the culture medium in another tube for later use (Step 6a).

Note: We tested different concentrations of Forskolin (1 μM, 5 μM, 10 μM), and we found that cells were healthy under 1 μM Forskolin treatment, while 5 μM and 10 μM seemed to have a toxic effect on the cells.

4. FFSS. The complete system includes: 125 mL glass bottle, pressure pump, rubber tubing system, flow rate indicator, precision FCS2/FCS3 cell booster chamber, and 40 mm cell culture slides (Figure 2).
   a. Check the airtightness of the FCS2/FCS3 system.
   b. Clean the FCS2/FCS3 system with 70% alcohol for 30 min, and then wash it with ddH_2O for 3 times, 10 min each time.
   c. Preheat 125 mL of FFSS loading buffer (RM) in a constant temperature water bath at 37°C.
   d. Assemble the FCS2/FCS3 system.
      i. Clamp the liquid outflow rubber tube and the balance air rubber tube.
      ii. Rinse the FCS2/FCS3 system with RM at high flow to remove air bubbles.
      iii. Loosen the clamped rubber tube to adjust the flow rate to the set value (14 mL/min).
   e. Clamp the rubber pipes at both ends of the cell booster chamber, take out the cell booster chamber and unscrew the cell booster chamber clockwise.
   f. Put the FCS2/FCS3 40 mm cell culture slides seeded with cells (with a 60 mm dish) into the cell booster chamber and tighten it.

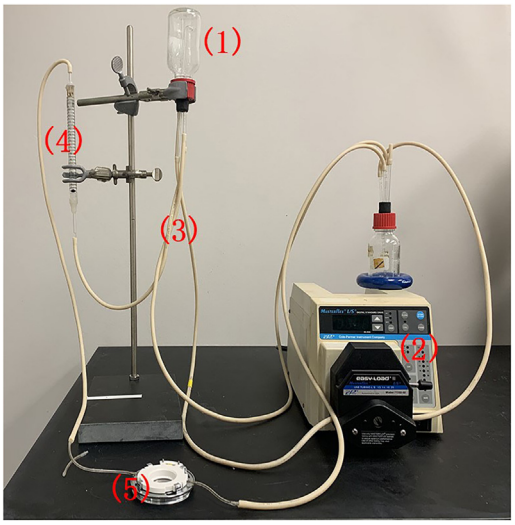


Figure 2. Instrument for fluid flow shear stress (FFSS)

(1) 125 mL glass bottle, (2) pressure pump, (3) rubber tubing system, (4) flow rate indicator, and (5) precision FCS2/FCS3 cell booster chamber.

g. Connect the rubber tubes at both ends to the cell booster chamber and loosen the clamped rubber tubes to operate the FCS2/FCS3 system normally.

Note: Gently tap the upper end of the cell force chamber to expel air bubbles in the cell force chamber.

h. Load cells with FFSS for 30 min (for FFSS issues see troubleshooting problem 4).
i. Upon completion, clamp the rubber pipes at both ends of the cell force chamber, and take out the cell culture slide in the cell force chamber for subsequent experiments.

5. Incubate primary lens fiber cells with GSH.
   a. Incubate lens primary fiber cells with 200 μL 1 mM GSH for 10 min in cell culture incubator (37°C, 5% CO_2).
   b. Wash cells three times with RM at RT, 5 min each time.
6. Treatment of lens primary fiber cells with H_2O_2 (More detail tips with H_2O_2 see troubleshooting problem 5).
   a. Add the previously collected and preserved cell culture medium (37°C) to the cells.
   b. Treat lens primary fiber cells with 50 μM H_2O_2 for 4 h (add on side of cell culture dish and gently mix).
   c. Wash cells three times with DPBS (RT), 5 min for each time.

Detect cell apoptosis and necrosis using Dead Cell Apoptosis Kit – Day 24

Timing: 1 h

The following experimental steps are used to confirm that PKA activator increase GSH transportation after HCs opening to protect lens primary fiber cells from apoptosis and necrosis.

7. Wash cells twice with cold BioLegend's Cell Staining Buffer.
8. Prepare FITC-Annexin V and PI mixed solution, 230 μL per lens primary fiber cell sample (200 μL Annexin V Binding Buffer, 10 μL Annexin V, 20 μL PI).
9. Add 230 μL of FITC-Annexin V and PI mixed solution to the cells and incubate for 20 min at room temperature (25°C) in the dark.
10. Wash cells three times with cold DPBS, 5 min for each time.

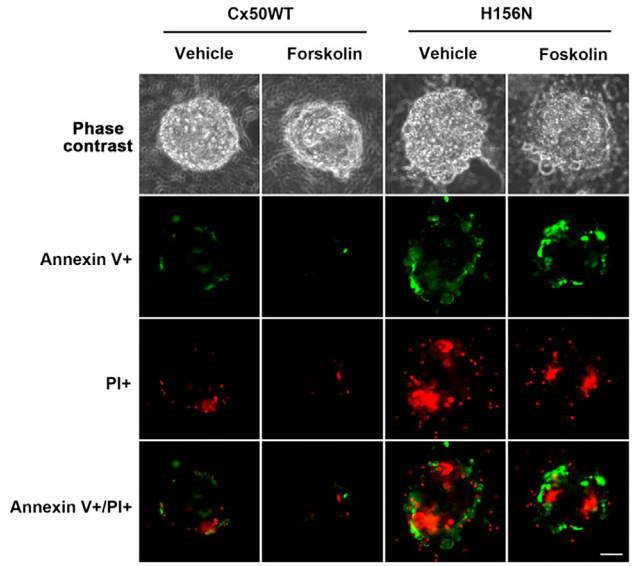


Figure 3. Apoptosis and necrosis of chicken lens primary cells infected with different retroviruses (RCAS(A)) with or without Forskolin

This figure is partially derived from a published article (Du et al., 2023), primary chick lens cell cultures were infected with high-titer recombinant RCAS(A) retroviruses containing Cx50, or Cx50 mutants H156N, treated with PKA activator, forskolin, and then subjected to fluid flow shear stress (FFSS) at 1 dyne/cm^2 for 30 min, incubated with 1 mM GSH for 10 min and treated with 50 μM H_2O_2 for 4 h. Cell apoptosis and necrosis were detected using a Dead Cell Apoptosis Kit. The green fluorescent signal is Annexin V, and the red fluorescent signal is PI. Scale bar: 50 μm.

11. Capture at least five microphotographs of fluorescence fields by a fluorescence microscope (Keyence BZ-X710) with a FITC and rhodamine filter (For imaging see troubleshooting problem 6).
12. Use the ratio of positive staining area to total area of lentoids to indicate the extent of cells under apoptosis or necrosis.

Note: All cells were photographed within 4 h to prevent fluorescence quenching.

EXPECTED OUTCOMES

Determine cells apoptosis and necrosis based on our previously described protocols (Quan et al.,[9] Shi et al; [3]). The FITC-Annexin V Apoptosis Detection Kit was used to detect the ratio of apoptosis/necrosis. The kit contains FITC-Annexin V, PI, and binding buffer. Healthy cell membranes are highly symmetrical, and phosphatidylserine is located on the inner surface of the cell membrane. If the cell is in the early apoptosis state, the cell membrane loses symmetry and causes phosphatidylserine to turn outward, FITC-Annexin V can bind to phosphatidylserine, showing green fluorescence. If the cell is necrotic, PI can enter the nucleus and combine with DNA, showing red fluorescence (Figure 3).

QUANTIFICATION AND STATISTICAL ANALYSIS

After staining, the fluorescence images are captured by a Keyence BZ-X710 All-in-One fluorescence microscope and analyzed. Since the lens cortical fiber cells "Lentoids" are round in shape, it is necessary to count the ratio of the Annexin V+ area to the total area and the ratio of the PI + area to the total area. In the NIH Image J software, we set thresholds for green fluorescence and red fluorescence, and the part exceeding the green threshold was regarded as Annexin V+, and the part exceeding the red threshold was regarded as PI+. Therefore, the ratio of lens cortical fiber cell apoptosis is: Annexin V+ area divided by the total area; the necrosis ratio is: PI + area divided by the total area. The average ratio of apoptotic/necrotic area of 5 fields of view is the ratio of apoptotic/necrotic area of one experiment. Each experiment was repeated 3 times (n = 3). The data is imported to GraphPad Prism software for graphical presentation and analysis.

LIMITATIONS

This protocol was designed to assess protection; therefore, it does not directly test HC function. The protocol involves the use of primary lens cells after altering expression of Cx50 HC and tests for cell death after H_2O_2 treatment. Notably, gap junction (GJ) function cannot be detected due to the cell morphology and growth mode in this particular set up. If it is needed to directly test the function of HC or GJ, researchers should use chicken embryonic fibroblast (CEF) and a dye uptake assay or scrape-loading dye transfer assay, not covered in this protocol, but further information can be found in Du et al.[10]

TROUBLESHOOTING

Problem 1

The extraction of primary lens cells failed, due to lack of the formation of ' Lentoids' by cell differentiation.

Potential solution

- Make sure that the eggs are incubated for no less than 10 days and no more than 11 days.
- Sanitize the eggs before conducting the experiment.
- Use all solutions as fresh as possible.

Problem 2

The growth status of the chicken lens primary cells is abnormal.

Potential solution

- Chicken lens primary cells are fairly sensitive, avoid frequent opening of the cell incubator during cell culture.
- Use ready-to-use cell culture medium.

Problem 3

Infection of chicken lens primary cells with retrovirus (RCAS(A)) is unsuccessful.

Potential solution

- When taking the retrovirus (RCAS(A)) out of the -80°C refrigerator and dissolving it, immediately add it to the medium containing the cells and mix well, avoid standing at room temperature for a long time.
- Double check infection efficiency using western blot.

Problem 4

Cells are washed away after FFSS.

Potential solution

- FCS2/FCS3 40 mm cell culture slides should be coated with 0.014% Type I collagen solution, this is conducive to the adherent growth of chicken lens primary cells.

Problem 5

Chicken primary lens cells death after H_2O_2 treatment.

Potential solution

- Because the lens primary cells are very sensitive to hydrogen peroxide, when adding H_2O_2, do not pipette the solution direct on top of the cells, but to the side wall of the cell culture dish, and mix quickly after adding.

Problem 6

Low fluorescence was detected of apoptotic/necrotic in lens primary cells.

Potential solution

- After using the FITC-Annexin V Apoptosis Detection Kit, take fluorescence pictures within 4 h to avoid fluorescence quenching.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Jean X. Jiang (jiangj@uthscsa.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

ACKNOWLEDGMENTS

The study was supported by NIH RO1 EY012085 (to J.X.J.) and F32DK134051 (to F.M.A.) and Welch Foundation grant AQ-1507 (to J.X.J.).

AUTHOR CONTRIBUTIONS

Y.D., S.G., and J.X.J. designed the study; Y.D. mainly conducted studies and performed experiments; Y.D. and Y.T. developed the protocol; Y.D., Y.Q. performed statistical analysis; Y.D., F.M.A., and J.X.J. wrote the manuscript; J.Z. provided photos; all authors critically revised and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Liu, J., Riquelme, M.A., Li, Z., Li, Y., Tong, Y., Quan, Y., Pei, C., Gu, S., and Jiang, J.X. (2020). Mechanosensitive collaboration between integrins and connexins allows nutrient and antioxidant transport into the lens. J. Cell Biol. *219*, e202002154.

2. Riquelme, M.A., Burra, S., Kar, R., Lampe, P.D., and Jiang, J.X. (2015). Mitogen-activated Protein Kinase (MAPK) Activated by Prostaglandin E2 Phosphorylates Connexin 43 and Closes Osteocytic Hemichannels in Response to Continuous Flow Shear Stress. J. Biol. Chem. *290*, 28321–28328.

3. Shi, W., Riquelme, M.A., Gu, S., and Jiang, J.X. (2018). Connexin hemichannels mediate glutathione transport and protect lens fiber cells from oxidative stress. J. Cell Sci. *131*, jcs212506.

4. Jiang, J.X. (2001). Use of retroviruses to express connexins. Methods Mol. Biol. *154*, 159–174.

5. Rath, A.L., Bonewald, L.F., Ling, J., Jiang, J.X., Van Dyke, M.E., and Nicolella, D.P. (2010). Correlation of cell strain in single osteocytes with intracellular calcium, but not intracellular nitric oxide, in response to fluid flow. J. Biomech. *43*, 1560–1564.

6. Jiang, J.X., and Goodenough, D.A. (1998). Retroviral expression of connexins in embryonic chick lens. Invest. Ophthalmol. Vis. Sci. *39*, 537–543.

7. Banks, E.A., Yu, X.S., Shi, Q., and Jiang, J.X. (2007). Promotion of lens epithelial-fiber differentiation by the C-terminus of connexin 45.6 a role independent of gap junction communication. J. Cell Sci. *120*, 3602–3612.

8. Jiang, J.X., Paul, D.L., and Goodenough, D.A. (1993). Posttranslational phosphorylation of lens fiber connexin46: a slow occurrence. Invest. Ophthalmol. Vis. Sci. *34*, 3558–3565.

9. Quan, Y., Du, Y., Wu, C., Gu, S., and Jiang, J.X. (2021). Connexin hemichannels regulate redox potential via metabolite exchange and protect lens against cellular oxidative damage. Redox Biol. *46*, 102102.

10. Du, Y., Tong, Y., Quan, Y., Wang, G., Cheng, H., Gu, S., and Jiang, J.X. (2023). Protein Kinase A Activation Alleviates Cataract Formation via Increased Gap Junction Intercellular Communication. iScience *26*, 106114.