



ELSEVIER

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method Article

Improved fluorescent signal in expansion microscopy using fluorescent Fab fragment secondary antibodies

David M. Sherry^{a,b,c,*}, Megan A. Stiles^{d,e}^a Department of Cell Biology, University of Oklahoma Health Sciences Center, United States^b Neurosurgery, University of Oklahoma Health Sciences Center, United States^c Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, United States^d Ophthalmology, University of Oklahoma Health Sciences Center, United States^e Dean McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, United States

A B S T R A C T

Expansion microscopy (ExM) is a microscopic imaging approach that can achieve super-resolution visualization of fluorescently labeled biological samples using conventional fluorescence microscopy. The method is based on embedding of a fluorescently labeled biological sample in a hydrogel matrix followed by the physical expansion of the specimen, which is then viewed using a conventional fluorescent microscope. Variations of the method can be used to visualize endogenously expressed fluorescent proteins, such as GFP, fluorescently tagged antibodies, nucleic acids, or other fluorescently tagged molecules. A significant challenge of the method is that the physical expansion of the specimen produces a concomitant reduction in fluorescence intensity, which can make imaging difficult. We describe an approach for amplifying fluorescence signal following expansion of immunolabeled tissue sections by applying fluorescently labeled Fab fragment secondary antibodies to intensify fluorescent signal and enhance detection of labeling using conventional fluorescent microscopy.

A method to increase immunofluorescence signal intensity of Expansion Microscopy specimens is described.

Method utilizes commercially available reagents.

Enhances ability to acquire useful images in expanded tissue samples.

© 2022 The Author(s). Published by Elsevier B.V.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

A R T I C L E I N F O

Method name: Fab secondary antibodies to improve fluorescent signal in ExM

Keywords: ExM, Fab fragment antibody, Immunohistochemistry

Article history: Received 14 June 2022; Accepted 20 July 2022; Available online 1 August 2022

* Corresponding author at: OUHSC, Department of Cell Biology, 940 SL Young Blvd. BMSB-101, Oklahoma City, OK 73104, USA.
E-mail address: david-sherry@ouhsc.edu (D.M. Sherry).

Specification table

Subject area;	Neuroscience
More specific subject area;	Microscopy
Name of your method;	Fab secondary antibodies to improve fluorescent signal in ExM
Name and reference of original method;	Protein Expansion Microscopy (ProExM) for Intact Tissues. Ref: Asano SM, Gao R, Wassie AT, Tillberg PW, Chen F, Boyden ES. 2018. Expansion Microscopy: Protocols for imaging proteins and RNA in cells and tissues. <i>Curr. Protoc. Cell Biol.</i> 80:e56 doi: 10.1002/cpcb56
Resource availability;	NA

Method details

Wide-field fluorescence microscopy and laser scanning confocal microscopy are workhorses for imaging macromolecules, cellular structure, and cellular processes in biological samples. However, the maximum resolution these microscopes can achieve is limited by the diffraction of light to approximately $0.25 \mu\text{m}$ in the horizontal (XY) plane and to roughly $0.6 \mu\text{m}$ in depth (Z-plane) [1], limiting the ability to resolve molecular complexes, organelles and other subcellular structures and domains. Advances in optical physics have led to the development of fluorescence-based light microscopy methods that can exceed the resolution barrier to achieve "super-resolution" in the range of 10–120 nm (XY) and 50–80 nm in the Z dimension [1–3]. These advances include Total Internal Reflectance Fluorescence (TIRF), Structured Illumination Microscopy (SIM), Stimulated Emission Depletion (STED), Photoactivated Localization Microscopy/Stochastic Optical Reconstruction Microscopy (PALM/STORM), and super-resolution spinning disk confocal microscopy, which all require specialized instrumentation. Another limitation of these approaches is that the depth of field can be restricted to a few hundred nanometers from the specimen surface.

Expansion Microscopy (ExM) provides an alternative approach to achieve super-resolution imaging in biological samples using conventional fluorescence microscopes, which are widely available. ExM relies on embedding fluorescently labeled cells or tissues anchored in a hydrogel matrix that is then isotropically expanded by a factor of up to 10X. Samples become optically transparent when expanded, permitting visualization of fluorescently labeled structures at super-resolution using conventional fluorescent microscopes [4–10]. A number of ExM variants that use different hydrogel matrices and labeling approaches have been developed for the visualization of a range of biological molecules including proteins, nucleic acids, and lipids [4–10]. Key challenges in ExM include crosslinking the target molecule(s) in the specimen to the hydrogel while preserving the ability to fluorescently label the target molecule, and the spatial "dilution" of the target molecule that occurs when the specimen is expanded. Several different matrices have been developed with specific chemistry tailored to crosslinking different types of target molecules into the hydrogel matrix prior to expansion of the specimen [4–10]. The spatial dilution of the target molecule that occurs during the physical expansion of the specimen cannot be prevented, as the target molecules within the specimen are physically moved away from one another, decreasing local concentration of the target and reducing signal intensity. For example, in X10 ExM the specimen is expanded by 10X in the X, Y, and Z dimensions, reducing the target molecule concentration by a factor of 10^3 , which is accompanied by a concomitant reduction in labeling intensity. This large reduction in signal intensity can render imaging of expanded samples difficult (Fig. 1). Another challenge, particularly for ExM procedures that utilize post-expansion immunolabeling, is that expanded samples are large and delivery of antibodies deep into the specimen can be difficult.

The approach described here employs monovalent, fluorescently-labeled Fab fragment secondary antibodies, which are much smaller than whole IgG antibodies, to improve delivery of secondary antibodies into expanded specimens and increase signal intensity (Fig. 2).

Materials and methods

Animals and tissue preparation

Long-Evans rats were maintained on a 12 h light:12 h dark light cycle (~25–40 lux at cage level) in a pathogen-free rodent barrier facility. Food and water were available ad libitum. Tissue preparation

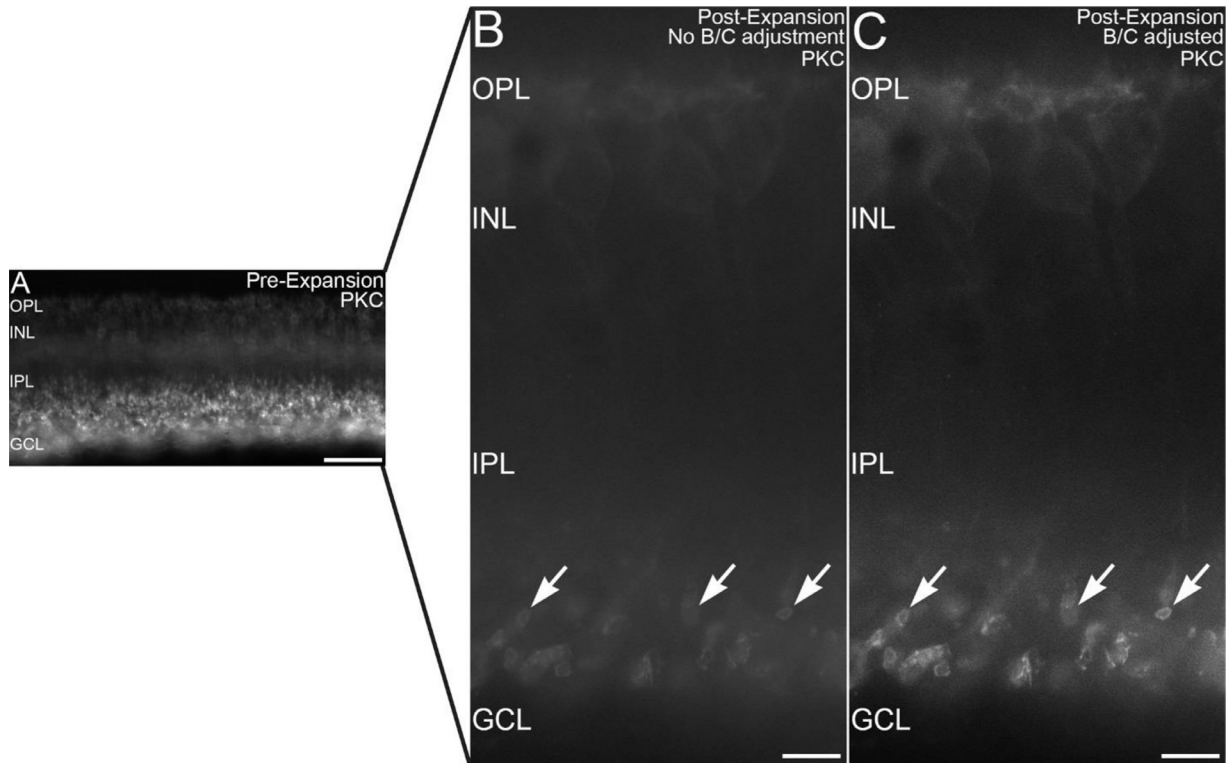


Fig. 1. ExM of pre-embedding immunolabeling for Protein Kinase C (PKC) in rod bipolar cells in rat retina. Wide-field images of PKC immunolabeling in 200 μm thick retinal sections acquired using a 10X objective lens before and after expansion (Expansion Factor = 4.09x). Immunolabeling intensity is decreased by expansion. (A) PKC immunolabeling before expansion. (B) After expansion (no adjustment of brightness and contrast). (C) Same image as panel B, with adjustment of brightness and contrast to highlight rod bipolar cell features. Arrowheads: rod bipolar cell synaptic terminals. B/C, brightness/contrast; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars = 100 μm.

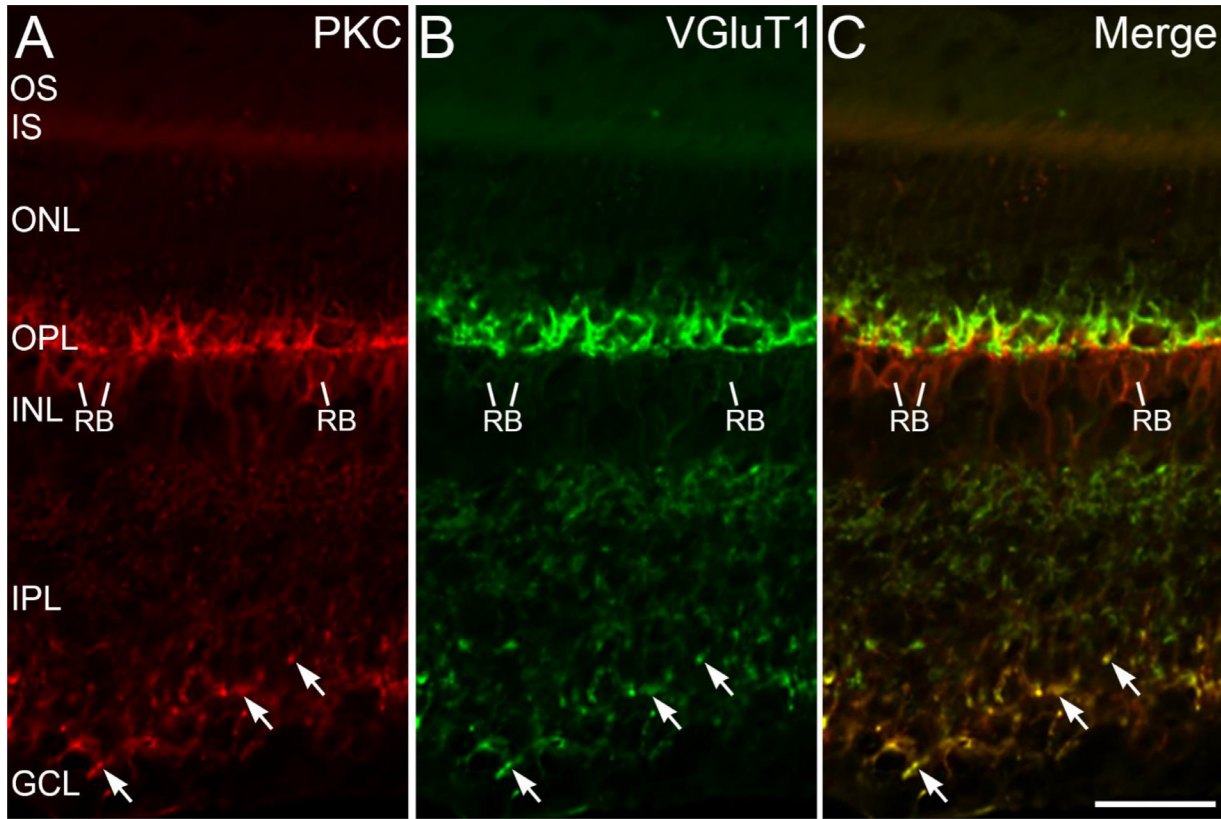


Fig. 2. Fab secondary antibodies produce strong signal in Post-ExM immunolabeling. Double labeling for Vesicular Glutamate Transporter 1 (VGLuT1) and Protein Kinase C (PKC) in rat retina. Images of post-expansion immunolabeling using Fab fragment secondary antibodies. Images acquired using a 20X objective lens. (A) PKC immunolabeling identifies rod bipolar cells (RB) in the inner nuclear layer (INL), their dendrites in the outer plexiform layer (OPL), and selectively labels their terminals (arrows) in the innermost portion of the inner plexiform layer (IPL), as expected. (B) Immunolabeling for VGLuT1 is present in the terminals of photoreceptors in the outer plexiform layer (OPL) and all types of bipolar cell terminals throughout the inner plexiform layer (IPL), as appropriate. (C) Overlay of panels A and B. ONL, outer nuclear layer; GCL, ganglion cell layer; IS, inner segments; OS, outer segments. Scale bar = 50 μ m.

followed typical procedures used for immunolabeling of retinal sections, as previously described [11]. Rats were anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg) by i.p. injection, then perfused with 0.1 M phosphate buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde in PBS for 20 min. Eyes were dissected free, the corneas were removed, and the resulting eyecups were fixed for one to two additional days in 4% paraformaldehyde in 0.1 M PBS or 0.1 M cacodylate buffer at 4°C. Eyecups were rinsed in PBS, cryoprotected using 30% sucrose in PBS, immersed in OCT medium (Sakura Tissue Tek; VWR, West Chester, PA), and frozen. Frozen sections (100–200 µm thickness) through the vertical meridian of the eyecup were cut and stored free-floating in PBS at 4°C for up to several days before immunolabeling.

Microscopy

Widefield images of expanded, immunolabeled retinal sections were imaged on an Olympus IX70 epifluorescence microscope fitted with filters for imaging standard green (AlexaFluor 488), near red (Alexafluor 568) and far red (AlexaFluor 647) fluorophores. Images were captured using 10X, 20X, and 40X objective lenses and a QiCAM CCD camera and Qcapture software (Qimage Vancouver, British Columbia, Canada). To prepare figures, image scale was calibrated, and images were imported into Photoshop software (Adobe Systems, Mountain View, CA). If necessary, adjustments were made to brightness and contrast to highlight specific labeling.

Procedure

The procedure below is adapted from the procedure for Protein Expansion Microscopy (ProExM) with post-expansion immunolabeling described by Asano et al. [7]. The reader is directed to Asano et al. [7] for formulations for the various reagents and solutions used to perform the ProExM procedure. For this report, we performed immunolabeling using well-characterized primary antibodies directed against well-known cell- and synapse-marker proteins in the mammalian retina: Vesicular Glutamate Transporter 1 (VGluT1), which labels the synaptic terminals of photoreceptors and bipolar cells [12,13], and Protein Kinase C α (PKC), which specifically labels rod bipolar cells [14]. Immunolabeling patterns for VGluT1 and PKC observed in ExM samples match those previously reported in the literature [12–14]. All primary and secondary antibodies used in these studies are available commercially (see Table 1).

Expansion procedure (adapted from the “ProExM” procedure described in ref [7])

Gelation:

Gelation requires the following solutions (for formulations see ref [7]): Acryloyl-X SE/DMSO stock (“AcX solution”); Monomer solution (“Stock X”); TEMED stock solution; Ammonium Sulfate stock solution; 4 hydroxy-TEMPO (4HT) solution; Alkaline buffer.

AcX infusion. Tissue sample placed in 0.1 mg/ml Acryloyl-X SE (AcX) in PBS (make by diluting AcX 1:100 with PBS), overnight at room temperature. No shaking.

Gelation. Prepare gelation chamber (see ref [7], Fig. 4) *before* removing tissue from AcX solution.

(1) Rinse tissue in PBS 2 × 15 min, RT.

Thaw components for Gelling solution (Monomer solution, 4HT solution, Ammonium Persulfate solution, TEMED solution). Keep in ice bath after thawing.

Add, in order, in a 47:1:1:1 ratio (Scale total volume amount to size of sample to embed. Need 400 µl of Gelling solution for a single coronal section of mouse brain):

- (a) Monomer solution
- (b) 4HT solution
- (c) TEMED solution
- (d) Ammonium Persulfate solution
- (e) Vortex briefly

Immediately place specimen into microfuge tube with gelling solution at 4°C in dark for 30 min. (in refrigerator).

Table 1

Primary and secondary antibodies.

Primary Antibodies	Dilution	Supplier
Protein Kinase C α (PKC) (rabbit polyclonal)	1:300	Sigma/Millipore. Cat# P4334; RRID:AB_477345
Vesicular Glutamate Transporter 1 (VGluT1; mouse monoclonal)	1:100	NeuroMab. Cat# 75-066, clone N28/9; RRID:AB_2187693
Vesicular Glutamate Transporter 1 (VGluT1; Guinea pig polyclonal)	1:1000	Synaptic Systems. Cat# 135 304, clone; RRID:AB_887878
<u>Secondary Goat anti-IgG Antibodies</u>		
Goat anti-Guinea Pig IgG AlexaFluor 488	1:100–1:200	Thermo/Molecular Probes. Cat# A11073; RRID:AB_2534117
Goat IgG anti-Mouse IgG AlexaFluor 488	1:100–1:200	Thermo/Molecular Probes. Cat# A11029; RRID:AB_138404
Goat IgG anti-Rabbit IgG AlexaFluor 568	1:100–1:200	Thermo/Molecular Probes. Cat# A11036; RRID:AB_10563566
<u>Secondary Fab Goat anti-IgG Antibodies</u>		
Fab Goat anti-Guinea Pig IgG-AlexaFluor 488	1:100–1:200	Jackson ImmunoResearch, Cat# 106-547-008; RRID:AB_2632435
Fab Goat anti-Mouse IgG-AlexaFluor 488	1:100–1:200	Jackson ImmunoResearch, Cat# 115-547-003; RRID:AB_2338869
Fab Goat anti-Rabbit IgG-AlexaFluor 568	1:100–1:200	Jackson ImmunoResearch, Cat# 111-547-003; RRID:AB_2338058

Important note: Perform the next steps at 4 °C within 5 min to prevent premature gelling.

After 30 min at 4 °C in fridge, place 20 μ l of gelling solution in gelation chamber sized to fit the specimen, and transfer section to chamber with soft paintbrush. Section must lie flat without wrinkles (use brush to remove wrinkles if necessary).

Add 20 μ l droplet of gelling solution to the lid (usually a coverslip) of the gelling chamber and slowly place the lid onto the chamber being sure to exclude air bubbles. (see ref [7], Fig. 4 for diagram).

Place gelation chamber in incubator at 37 °C for 2 h. Gelation chamber must be level.

Remove specimen from incubator, gently remove gelation chamber top with a razor blade, remove chamber sides, and trim off excess gel.

Wet a paintbrush with Alkaline Disruption Buffer solution and wet the gel top and sides. Wait until buffer soaks the border of the gel, then gently coax the specimen off the glass slide using soft paintbrush.

Place specimen in Alkaline Disruption Buffer Solution at room temperature for 15 min x 2.

Place specimen, into 50 ml conical tube lid filled with Disruption Buffer heated to just short of boiling. Place tube lid with specimen into a polyethylene container with wet paper towels on the bottom and a loose fitting lid. Place the container holding the specimens into a 50 °C oven with humid atmosphere for 5 h.

Remove specimens from oven and allow specimens to cool to room temperature. Specimens must remain covered with Alkaline Disruption Buffer as they cool.

Trim off excess gel using a razor blade and rinse samples in 1x PBS, 3 \times 5 min at room temperature.

Immunolabeling (note: specimen may continue to expand during this step):

Remove PBS and apply blocker solution (5% normal goat serum + 2.5% BSA + 0.1% triton X in PBS) for 2 h at room temperature.

Remove blocker and replace with desired primary antibodies diluted in blocker solution (See Table 1). Incubate overnight to 24 h at room temperature with gentle rocking.

Remove primary antibody and rinse in PBS, 4 \times 30 min at room temperature. Gentle rocking can be used.

Apply fluorescently labeled Fab fragment secondary antibodies diluted 1:100 in blocker solution. Incubate overnight to 24 h at room temperature with gentle rocking.

Remove secondary antibody and rinse in PBS, 4 × 30 min at room temperature. Gentle rocking can be used.

Mounting specimen for imaging

Use Poly-L-Lysine to anchor the specimen to the coverslip to prevent movement during imaging.

- (a) Clean a 22 x 50 mm coverslip with water, then 100% ethanol. Air dry.
- (b) Soak the coverslip with 0.1% poly-L-lysine in water (w/v) for 20 min at room temp.
- (c) Rinse coverglass surface 3x with water
- (d) Air dry (covered to prevent dust accumulation).

Transfer tissue specimen in expanded gel to the poly-L-Lysine-coated coverslip. Because fade-retardant mounting media can cause tissue shrinkage and distortion, no fade-retardant mounting medium was used [7].

- (a) Remove most of the rinse liquid from the vessel holding the labeled, expanded specimen (i.e., petri dish, etc.).
- (b) Place a clean coverslip (not poly-lysine coated) next to expanded specimen and use paint brush to gently transfer the specimen onto the coverslip.
- (c) Lift the coverslip with the specimen out of its container using a small paintbrush
- (d) Gently wick away liquid on the expanded specimen's top surface using a kimwipe then from the sides of the gel, and finally from under the specimen.
- (e) Use paintbrush to gently slide the expanded specimen from the clean coverslip to the Poly-L-Lysine coated coverslip. Mount the specimen onto a clean microscope slide. The side of the specimen on the Poly-L-Lysine coated coverslip will be the side to image for best quality imaging.
- (f) After mounting, add a small amount of 1X PBS to keep the gel hydrated to prevent shrinkage.

Imaging

Place specimen onto the microscope and capture images. Imaging can be performed using wide-field epifluorescence or confocal microscopes, according to availability and experimental needs.

Troubleshooting

Imaging shortly after completing the labeling procedure is strongly recommended as fluorescent signal in expanded samples tends to decay with time. If post-imaging storage of the specimen is desired, the gel can be removed from the coverslip and stored in 1x PBS at 4 °C covered in foil. A further recommendation is to minimize exposure of the specimen to fluorescent excitation to reduce bleaching of fluorescent tags. Many fade-retardant mounting media can cause tissue shrinkage, which is not desirable for ExM.

Pre-embedding methods for immunolabeling of specimens for ExM have been used very successfully [4–7,9,10]. However, pre-embedding ExM procedures, such as enzymatic digestion steps, can affect the antigen(s) of interest as well as primary and secondary antibodies bound into the specimen, leading to reduced signal [4–7,9,10]. Digestion procedures also can affect the antigen(s) of interest in post-embedding ExM, diminishing binding of primary antibodies to the antigen of interest and reducing signal. Careful adjustment of digestion and disruption procedures may be required to optimize antigen retention in both pre- and post-expansion ExM preparations. A variety of embedding matrices and cross-linking reagents have been developed to improve retention of specific types of molecules in ExM preparations [4–10], and careful matching of the target molecule to the matrix and cross-linking procedures also may improve labeling. Although examples shown in this report illustrate single and double-immunolabeling, ExM is compatible with triple immunolabeling and additional

fluorescent tags, such as DAPI to label nuclei. However, some fluorescent labels do not tolerate pre-embedding ExM procedures well, leading to weak or absent fluorescent signal. For example, the popular deep-red tag AlexaFluor647 is sensitive to pre-embedding ExM procedures, and use of other deep-red tags, such as Biotium 633, may be preferable [9,10].

Although Fab fragment antibodies have only one antigen binding site, that does not guarantee the absence of cross-reactivity of the Fab antibody. It is recommended that all secondary antibodies used for ExM labeling, regardless of whether they are Fab fragments or whole IgGs, be highly cross-adsorbed to remove non-specific cross-species reactivity.

The procedure described here was used specifically for post-expansion immunolabeling of relatively thick tissue sections. We found that the use of Fab fragment secondary antibodies, which are much smaller than whole IgG secondary antibodies, improved the fluorescent signal in post-expansion ExM. The use of Fab fragment secondary antibodies also should be useful for enhancing labeling intensity in ExM of cultured cell preparations.

Ethics statements

Rats of both sexes were used in these studies. No association of sex on results was noted. All animal procedures were approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee. Procedures conformed to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978), US Public Health Service guidelines, and the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Declaration of Competing Interests

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.

CRedit authorship contribution statement

David M. Sherry: Conceptualization, Methodology, Resources, Writing – original draft, Visualization. **Megan A. Stiles:** Methodology, Investigation, Writing – review & editing.

Acknowledgments

Funding: Research reported in this publication was supported in part by 1P20GM125528 “Cellular and Molecular GeroScience CoBRE” from the National Institute of General Medical Sciences.”

The monoclonal antibody against VGlut1 (clone N28/9) was developed by and/or obtained from the UC Davis/NIH NeuroMab Facility, supported by NIH grant U24NS050606 and maintained by the Department of Neurobiology, Physiology and Behavior, College of Biological Sciences, University of California, Davis, CA 95616.

References

- [1] R.S. Fischer, Y. Wu, P. Kanchanawong, H. Shroff, C.M. Waterman, Microscopy in 3D: a biologist's toolbox, *Trends Cell Biol.* 21 (12) (2011) 682–691 Oct 31.
- [2] T. Azuma, T. Kei, Super-resolution spinning-disk confocal microscopy using optical photon reassignment, *Opt. Express* 23 (11) (2015) 15003–15011 Jun 1.
- [3] G. Jacquemet, A.F. Carisey, H. Hamidi, R. Henriques, C. Leterrier, The cell biologist's guide to super-resolution microscopy, *J. Cell Sci.* 133 (11) (2020) Jun 11, doi:10.1242/jcs.240713.
- [4] F. Chen, P.W. Tillberg, E.S. Boyden, Expansion Microscopy, *Science* 347 (6221) (2015) 543–548 Jan 30.
- [5] T.J. Chozinski, A.R. Halpern, H. Okawa, H.J. Kim, G.J. Tremel, R.O.L. Wong, J.C. Vaughn, Expansion microscopy with conventional antibodies and fluorescent proteins, *Nat. Methods* 13 (6) (2016) 485–488 Apr 11.
- [6] P.W. Tillberg, F. Chen, K.D. Piatkevich, Y. Zhao, C.C.J. Yu, B.P. English, L. Gao, A. Martorell, H.J. Suk, F. Yoshida, E.M. DeGennaro, D.H. Roossien, G. Gong, U. Seneviratne, S.R. Tannenbaum, R. Desimone R, D. Cai, E.S. Boyden, Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies, *Nat. Biotechnol.* 34 (9) (2016) 987–992 July 4.

- [7] S.M. Asano, R. Gao, A.T. Wassie, P.W. Tillberg, F. Chen, E.S. Boyden, Expansion microscopy: protocols for imaging proteins and RNA in cells and tissues, *Curr. Protoc. Cell Biol.* 80 (2018) e56 Aug 2, doi:10.1002/cpcb56.
- [8] D.E. Sun, X. Fa, Y. Shi, H. Zhang, Z. Huang, B. Cheng, Q. Tang, W. Li, Y. Zhu, J. Bai, W. Liu, Y. Li, X. Wang, X. Lei, X. Chen, Click-ExM enable expansion microscopy for all biomolecules, *Nat. Methods* 18 (1) (2020) 107–113 Dec 7.
- [9] S. Truckenbrodt, M. Maidorn, D. Crzan, H. Wildhagen, S. Kabatas, S.O. Rizzoli, X10 expansion microscopy enables 25-nm resolution on conventional microscopes, *EMBO Rep.* 19 (9) (2018) e45836 Sep, doi:10.15252/embr.201845836.
- [10] S. Truckenbrodt, C. Sommer, S.O. Rizzoli, J.G. Danzl, A practical guide to optimization in X10 expansion microscopy, *Nat. Protocols* 14 (3) (2019) 832–863 Mar.
- [11] M.P. Agbaga, M.A. Stiles, R.S. Brush, M.T. Sullivan, A. Machalinski, K.L. Jones, R.E. Anderson, D.M. Sherry, The Elov14 spinocerebellar ataxia-34 mutation 736T>G (p.W246G) impairs retinal function in the absence of photoreceptor degeneration, *Mol. Neurobiol.* 57 (11) (2020) 4735–4753 Aug 11.
- [12] J. Johnson, N. Tian, M.S. Caywood, R.J. Reimer, R.H. Edwards, D.R. Copenhagen, Vesicular neurotransmitter expression in developing postnatal rodent retina: GABA and glycine precede glutamate, *J. Neurosci.* 23 (2) (2003) 518–529 Jan 15.
- [13] D.M. Sherry, M.M. Wang, J. Bates, L.J. Frishman, Expression of vesicular glutamate transporter 1 in the mouse retina reveals temporal ordering in development of rod vs. cone and ON vs. OFF circuits, *J. Comp. Neurol.* 465 (4) (2003) 480–498 Oct 27.
- [14] S. Haverkamp, H. Wässle, Immunocytochemical analysis of the mouse retina, *J. Comp. Neurol.* 424 (1) (2000) 1–23 Aug 14.