

Video Article

Correlative Super-resolution and Electron Microscopy to Resolve Protein Localization in Zebrafish Retina

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Abstract

We present a method to investigate the subcellular protein localization in the larval zebrafish retina by combining super-resolution light microscopy and scanning electron microscopy. The sub-diffraction limit resolution capabilities of super-resolution light microscopes allow improving the accuracy of the correlated data. Briefly, 110 nanometer thick cryo-sections are transferred to a silicon wafer and, after immunofluorescence staining, are imaged by super-resolution light microscopy. Subsequently, the sections are preserved in methylcellulose and platinum shadowed prior to imaging in a scanning electron microscope (SEM). The images from these two microscopy modalities are easily merged using tissue landmarks with open source software. Here we describe the adapted method for the larval zebrafish retina. However, this method is also applicable to other types of tissues and organisms. We demonstrate that the complementary information obtained by this correlation is able to resolve the expression of mitochondrial proteins in relation with the membranes and cristae of mitochondria as well as to other compartments of the cell.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56113/>

Introduction

Methods to determine the subcellular localization of proteins and their relationship to different compartments of the cell are essential tools to understand their functions and possible interactions. Super-resolution microscopy in combination with electron microscopy provides such information¹. Ground state depletion microscopy followed by individual molecule return (GSDIM) is a super-resolution microscopy technology compatible with a wide range of organic and genetically encoded fluorophores² and achieves a lateral resolution up to 20 nm³. The incorporation of methods with higher resolution than standard diffraction-limited microscopy improves the accuracy of the correlation^{4,5,6}. In order to achieve the best correlation of protein expression with a specific subcellular compartment and to reduce the volume of uncertainty⁷ the use of the same ultrathin section for light and electron microscopy is recommended. Among the different sectioning methods, Tokuyasu cryo-section protocol does not require dehydration or resin embedding and, in addition, preserves the antigenicity of many epitopes and provides good tissue ultrastructure⁸. Several methods have demonstrated the applicability of these sections in correlative light and electron microscopy (CLEM)^{4,5,9,10}.

The zebrafish retina is a valuable model to study visual development and human disease mechanisms given its highly conserved structure and function across vertebrates. In particular, retinal photoreceptors display the same architecture as mammalian photoreceptors, with a basal synapse, an apico-basally elongated nucleus, clustering of mitochondria in the more apical inner segment and an outer segment composed of membrane disks in the most apical position¹¹. Protein localization to the diverse cellular compartments is conserved between zebrafish and human, allowing investigation of the biological function of human disease-relevant proteins^{12,13}.

Here we present a protocol to prepare larval zebrafish retina samples to resolve the localization of the mitochondrial outer membrane protein Tom20 by correlative super-resolution light and electron microscopy. The method is based on collecting cryo-sections on silicon wafers and obtaining contrast by topographical information produced after application of a thin layer of platinum. These steps are clear technical improvements in terms of ease of use, reproducibility, and time to complete experiments. We have recently demonstrated the applicability of the method to detect nuclear pores and mitochondrial proteins in mouse tissue¹⁴.

Protocol

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local authorities.

1. Preparation of Ultrathin Sections on Silicon Wafers

1. Sample fixation
 1. Prepare the fixative solution containing 0.1% glutaraldehyde, 4% formaldehyde in 0.1 M cacodylate buffer.
NOTE: **CAUTION!** Use caution when working with glutaraldehyde, formaldehyde and cacodylate buffer, wear appropriate personal protective equipment and work in a fume hood.
 2. Euthanize 5 days post fertilization (5 dpf) zebrafish larvae with tricaine (ethyl 3-aminobenzoate methanesulfonate, 0.4% w/v in PBS (phosphate buffer saline, pH 7)) as previously described¹⁵.
 3. Fix the larvae by immersion in pre-chilled fixative solution on ice. Remove the head and incubate overnight at 4 °C with gentle rocking.
 4. Dissect the eyes by trimming the tissue around the eye using fine forceps and a microdissection scalpel (under a binocular) in chilled fixative solution on a 1% agarose-bedded plate. Transfer the eyes to a tube with fresh pre-chilled fixative.
 5. Wash twice in PBS (phosphate buffer saline, pH 7.4) for 5 min each wash at room temperature (RT).
2. Gelatin infiltration and mounting
 1. Warm up 15 mL local food brand gelatin 12% w/v in PB (phosphate buffer, 0.1 M pH 7.4) at 40 °C. Remove the PBS and add gelatin solution to the tubes containing the eyes. Tap the tube gently to ensure the gelatin infiltrates the sample and incubate for 10 - 30 min at 40 °C in a thermoblock with gentle shaking or in a water bath.
 2. Fill 12 mm x 5 mm x 3 mm silicon or polyethylene flat embedding molds with warm gelatin in a 40 °C water bath. Add two eyes per mold using a pipette, align them properly under a binocular using a dissection needle and let the gelatin cool down at room temperature for 1 min and harden at 4 °C for 20 min.
 3. Re-trim the gelatin block under the binocular to fit one eye per block using a razor blade.
 4. Transfer the gelatin embedded eyes to 2.3 M sucrose in PB on ice. Incubate at 4 °C overnight.
 5. Exchange to new 2.3 M sucrose solution and store at 4 °C or -20 °C; after this step, samples are ready for sectioning or can be stored at -20 °C for several weeks to months.
 6. Re-trim the gelatin block to almost the size of the eye before transferring to a cryo-pin. Freeze in liquid nitrogen and transfer to a cryo-ultramicrotome.
3. Cryosectioning
 1. Cut 110 nm thick-sections at -120 °C with a diamond knife in the cryo-ultramicrotome.
 2. Pick the sections with a wired loop containing a droplet of 2% methylcellulose (in water) and 2.3 M sucrose solution (1:1). Transfer sections to a 7 mm x 7 mm silicon wafer. Store sections at 4 °C until further processing.

2. Immunolabelling

1. Wash wafers with PBS at 0 °C for 20 min on four drops by placing the wafers upside down on the drops. Wash in PBS 2x 2 min at room temperature.
2. Incubate 3 times in 0.15% glycine in PBS, for 1 min each. Wash 3x for 1 min/wash with PBS. Pre-incubate with PBG (PBS with 0.5 % Bovine Serum Albumin BSA and 0.2 % Gelatin type B) for 5 min.
3. Incubate with rabbit anti-Tom20 (see the **Table of Materials**) in PBG (4 µg/mL) for 30 min at room temperature.
4. Wash 6x for 1 min/wash in PBG. Pre-incubate with PBG for 5 min at RT. Incubate with anti-rabbit Alexa 647 F(ab')₂ (see the **Table of Materials**) in PBG (7.5 µg/mL) for 30 min.
5. Wash 6x for 1 min/wash in PBG. Wash 3x 2 min in PBS. Incubate with DAPI (4 µg/mL) in PBS for 10 s. Wash 2x 2 min in PBS.

3. Super-resolution Microscopy

1. Incubate the wafer briefly (10 s) by placing it on a droplet of a 1:1 solution of glycerol (80%) and imaging buffer containing an oxygen scavenging system (200 mM Phosphate buffer containing 10% glucose, 0.5 mg/mL glucoseoxidase, 40 µg/mL catalase, 15 mM beta-mercaptoethylamine hydrochloride (MEA HCL), pH 8.0).
2. Transfer the wafers (section facing down) to a glass bottom (thickness 170 ± 5 µm) Petri dish onto a fresh drop of the 1:1 mixture of glycerol (80%) and imaging buffer (as in step 3.1).
3. Remove from all sides, with a pipette, most of the liquid underneath the wafer. Use silicone stripes to fix the wafer to the bottom of the Petri dish.
NOTE: Silicone stripes are made out of two-component silicone-glue (3 mm x 12 mm).
4. Image sections on an inverted microscope using a high numerical aperture (e.g. 160X / NA 1.43; see the **Table of Materials**) oil immersion super resolution dedicated objective. Prior to imaging, let the sample equilibrate to microscope temperature in order to minimize/reduce lateral and axial drift.
5. Center the area of interest and acquire first widefield epifluorescence reference images. Change to the super resolution operation mode. Adjust the exposure time of the camera to 15 ms and set the electron multiplying (EM) gain to the maximum of 300.
6. Illuminate sample with the 642 nm continuous wave laser at maximum laser power (corresponding to ~2.8 kW/cm²) in epifluorescence mode. As soon as the single molecule blinks are well separated in each frame, so that the probability is low that individual signals overlap, set the laser power to ~0.7 kW/cm². Record the raw image in epifluorescence mode by acquiring a minimum of 30,000 frames.

NOTE: All these parameter can vary depending on different specimens and labeling densities.

7. From the raw data, generate a reconstruction event list (localizations of each single molecule blink in the raw image) using a detection threshold of 30 photons (needs to be adjusted according to the sample) by clicking "Evaluate" in the 't-series analysis' under 'Tools'.
8. Visualize the super resolution image by Gaussian fitting¹⁶ applying a rendering pixel size of 4 nm by clicking "Create Image" in the 'event list processing panel' under 'Tools.'

NOTE: For generating the super resolved image the integrated software tools of the system used in this study were employed. However, the described super resolution imaging could be performed on any other single molecule localization based system, and the data could be processed with open source software tools as thunderSTORM¹⁷.

4. Platinum Shadowing

1. Remove silicon stripes and add a drop of PBS close to the edges of the wafer to lift it up from the Petri dish. Wash the wafer 2x 2 min in PBS, then post fix it with 0.1% glutaraldehyde in PBS for 5 min. Wash 2x for 2 min/wash in PBS.
2. Incubate the wafer twice for 5 min/incubation in 1 drop of methylcellulose 2 % in water on ice. Insert the wafer in a centrifuge tube and centrifuge at 14,100 x g for 90 s. Mount it on a SEM aluminum stub with conducting carbon cement.
3. Add a layer of 2 to 10 nm of platinum/carbon on the sample by rotary shadowing at 8° using an electron beam evaporation device settings: 1.55 kV, 55 mA, at 0.3 nm/s, and an angle of 8°, rotation level 4.

5. Scanning Electron Microscopy

1. Image sections with a scanning electron microscope at 1.5 kV, 2 mm working distance and with an In lens secondary electron detector.

6. Alignment of Light and Electron Microscopy Images

1. Open both types of images with Fiji¹⁸ by clicking "File|Open". Adjust canvas size by clicking "Image| Adjust| Canvas size" and bring both images to a stack by clicking "Image| Stacks| Images to stack". Save the stack as tiff file type.
2. In Fiji, open a new TrackEM2¹⁹ interface by clicking "File|new|TrackEM2 (new)". Import the stack with both images by right clicking on the black window and selecting "Import stack".
3. Align light microscopy image to electron microscopy image manually with landmarks by using a right mouse click on the image and selecting "Align|Align layer manually with landmarks".
 1. Pick the select tool (the black arrow) to add landmarks. Use the shape of nuclei as reference to select the same edges in both images (**Supplementary Figure 1**). Add several points (minimum three points need to be selected). Apply alignment with an affine model by right mouse clicking, and selecting "Apply transform| Affine model".
4. Change layer transparency (see **Supplementary Figure 1**) to assess the quality of the alignment.

Representative Results

The expression of the protein Tom20, a subunit of the translocase mitochondrial outer membrane complex²⁰, was determined, in thin sections of larval zebrafish retina, by super-resolution light microscopy (**Figure 1**) and this information was complemented with the topographical signal obtained by scanning electron microscopy after platinum shadowing of the same sections. These correlative data confirm the localization of a protein in association with a particular compartment, the outer mitochondrial membrane and, in addition provide information about the relation of the protein with other organelles of the cell.

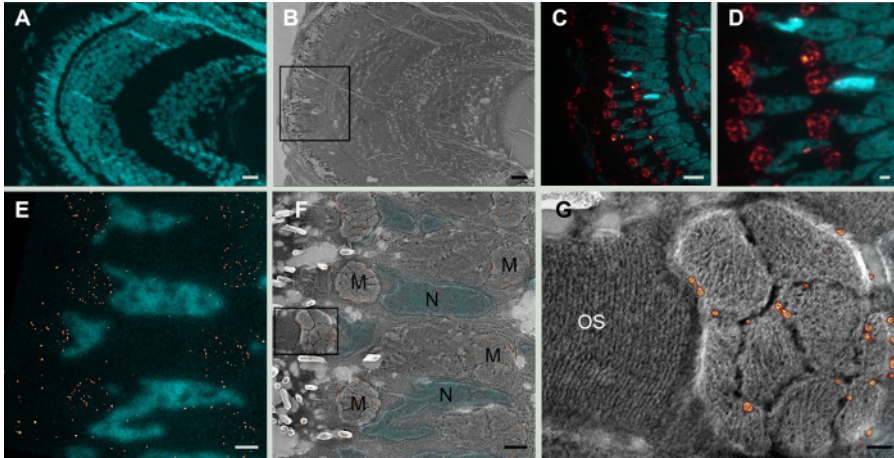


Figure 1: CLEM on zebrafish retina. **A.** Low magnification widefield image of a 5 dpf zebrafish retinal section, nuclei stained with DAPI (cyan). **B.** Scanning electron microscopy of the same area. **C.** Higher magnification widefield image of frame in B. Nuclei stained with DAPI (cyan) and Tom20 mitochondrial staining appears in red. **D.** Widefield image of same section at higher magnification. The pattern of Tom20 expression is at the clusters of mitochondria. **E.** Expression of Tom20 (red dots) detected by GSDIM microscopy. Nuclei stained with DAPI (cyan). **F.** Same section as E combining correlative super-resolution and scanning electron microscopy. Tom20 staining (red dots) appears at the mitochondrial cluster (M) at the outer membranes of mitochondria. Fluorescence DAPI signal in the nuclei (N) corresponds with the topography of the SEM image. **G.** High magnification image of frame in F. The scanning electron microscopy image provides context to the GSDIM image (red dots). Mitochondrial cristae are clearly visible and the Tom20 staining is localized to the outer membranes of mitochondria. The membranes of the outer segment the photoreceptors (OS) are clearly resolved. Image pixel size 5 nm. Scale bars: A, B, and C: 10 μ m; D: 2 μ m; E and F: 1 μ m and G: 0.2 μ m. [Please click here to view a larger version of this figure.](#)

Supplementary Figure 1: Alignment of light and electron microscopy images. **A.** Screenshot from TrackEM2 interface with SEM image and numbered landmarks (yellow) along different nuclei. **B.** Screenshot from TrackEM2 interface with fluorescence image and numbered landmarks (yellow) along different DAPI stained nuclei. To change layer transparency the sliders on the left upper part of the menu can be used. Scale bar: 1 μ m. [Please click here to download this file.](#)

Discussion

This method combines super-resolved protein localization with context information to determine the precise position of proteins in an organelle. We demonstrate here the completion of the experiment to visualize the expression of Tom20 in the outer membrane of mitochondria, and its relation to other organelles like nuclei or outer segments of the photoreceptor in the larval zebrafish retina.

Tokuyasu cryo-sectioning requires some training to acquire well-preserved sections. However, this is a method employed in many laboratories with demonstrated success²¹. Transfer of the sections to the silicon wafer is very simple and no special considerations are needed. The use of glycerol in the imaging buffer is a very critical step to avoid drying of the sections. For super-resolution imaging the best results are obtained when the wafer is very close to the glass bottom of the Petri dish. The silicone stripes help maintaining the wafer in this position. Special care has to be taken while removing the stripes in order to avoid damaging the sections.

The thickness of the cryo-sections, around 100 nm, thinner than the optical resolution in the Z-dimension, additionally facilitates the accuracy of this correlative method as the super-resolution microscopy signal is coming only from this thin layer and the scanning electron microscope signal is displaying the topography of the sample. This method could also be combined with multicolor imaging. However, special care must be taken that the sample can be imaged under same conditions (e.g. imaging buffer) and cross talk should be prevented.

One limitation of the method is its two dimensional approach, since only a limited number of serial sections (about 3 to 7 per wafer) can be collected. Thus, projects dealing with volume analysis would not be ideal. However, it is a method that can be applied to detect protein expression in any type of tissue by simple sectioning of the sample. We provide a method without use of classical contrast agents like uranyl acetate or lead citrate. The contrast by platinum shadowing provides very informative topographical contrast, but in some cases membranes are difficult to resolve. This may pose problems for projects that need, for example, to determine the expression of proteins in small vesicles.

Our protocol, based on Tokuyasu cryo-sections, uses standard equipment to obtain correlative results from super-resolution and scanning electron microscopes. The collection of sections on silicon wafers and the use of platinum for contrast are simple steps to provide stability and reproducibility to the sample preparation.

Disclosures

The authors have nothing to disclose.

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