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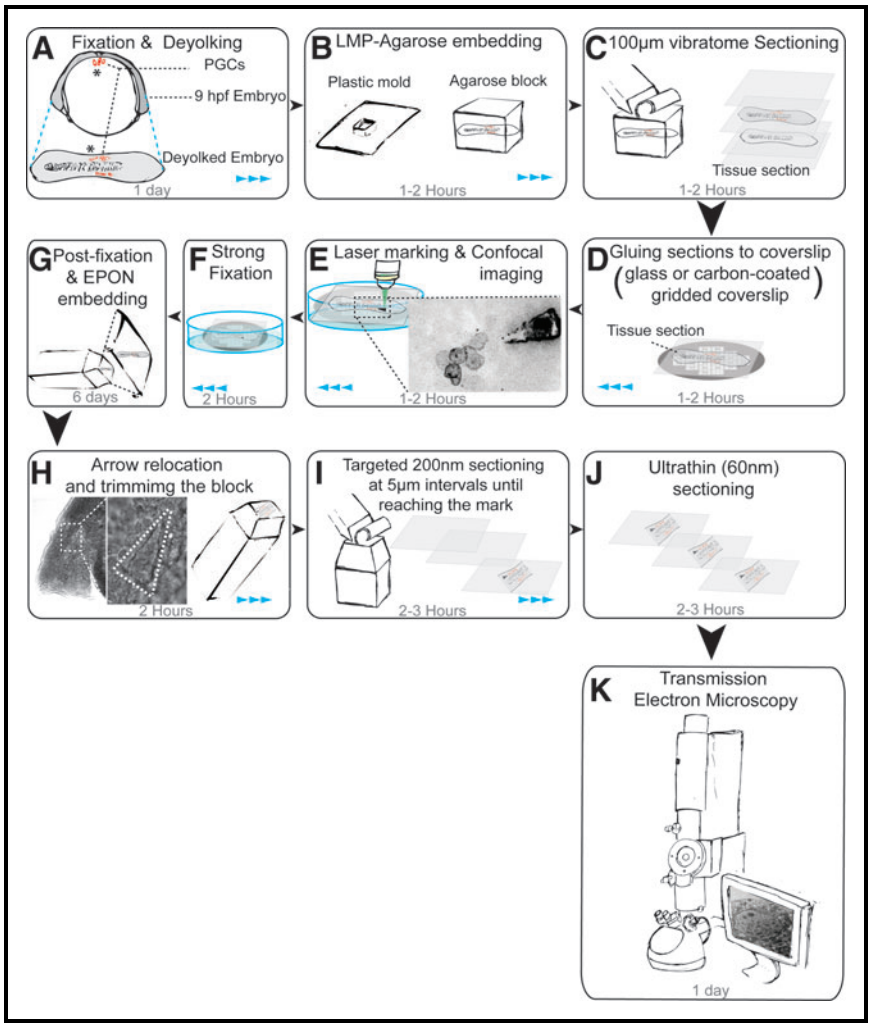
Correlative Light and Electron Microscopy of Rare Cell Populations in Zebrafish Embryos Using Laser Marks

Mohammad Goudarzi,[1] Karina Mildner,[2] Felix Babatz,[3] Dietmar Riedel,[4] Christian Klämbt,[3] Dagmar Zeuschner,[2] and Erez Raz[1]



[1]Institute of Cell Biology, ZMBE, University of Münster, Münster, Germany.
[2]Electron Microscopy Unit, Max Planck Institute for Molecular Biomedicine, Münster, Germany.
[3]Institute for Neurobiology, University of Münster, Münster, Germany.
[4]Electron Microscopy Group, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany.

Correlative light and electron microscopy techniques are powerful tools that allow the identification and analysis of fluorescently labeled cells and structures of interest by an electron microscope (EM).[1–3] Nevertheless, employing the methods listed above for the identification of rare cell populations can be very time consuming.[4] A recently described method combines imaging of a fluorescently labeled tissue before fixation, with laser etching at the region of interest after embryo fixation.[5] While the region of interest in the zebrafish embryo could be better focused on, this technique does not assist the identification of rare cell populations or specific cellular compartments when adjacent morphological landmarks are not present. In this study, we present a procedure based on a recently described method[6] that allows effective identification and EM analysis of a small group of cells within the embryo, without relying on distinct morphological markers (Fig. 1). We demonstrate the merit of this procedure using zebrafish primordial germ cells, a population of less than 25 cells within early embryos, which at that developmental stage (8–12 hours post fertilization [hpf]) consist of more than 20,000 cells.[7,8] This method relies on preservation of the fluorescent protein signal after fixation, combined with two-photon laser marking within the tissue at defined locations relative to the position where the cells of interest reside.

Concise Workflow

Collect and fix embryos (8–12 hpf in this study) whose cells or subcellular structures of interest are fluorescently labeled (with GFP, YFP, or mCherry), dechorionate the embryos,[9] and fix with 2% PFA + 0.2% Glutaraldehyde in a 0.1 M PHEM buffer (60 mM of PIPES, 25 mM of HEPES, 2 mM $MgCl_2$, 10 mM of EGTA), pH 6.9 at room temperature in a glass dish.

- After 10 min of fixation, deyolk the embryos under a fume hood as shown in Supplementary Movie S1 (Supplementary Data are available online at www.liebertpub.com/zeb).
- Continue fixation for 2 h at room temperature.
- Transfer to 1%PFA in the 0.1 M PHEM buffer using a glass pipette and select embryos for embedding based on the fluorescence signal (Fig. 1A).

Embedding of embryos in 4% low melting point (LMP) agarose in small plastic molds (Fig. 1B and Supplementary Data) and section to 100-μm-thick slices using a vibratome.

Collection of slices into flat-bottom plates (e.g., Corning® Costar® cell culture plates) that contain the 0.1 M PHEM buffer and screening for the slices containing fluorescently labeled cells [e.g., using a low magnification on an epifluorescence microscope (Fig. 1C)].

Gluing the selected slices to glass or carbon-coated gridded coverslips (the latter that can be produced or purchased, help to localize the region of interest when trimming the block) using a drop of LMP agarose (Fig. 1D). Transfer the samples to 0.1 M PHEM buffer.

FIG. 1. Schematic workflow of the correlative light and electron microscopy using laser marking. **(A)** Zebrafish embryo fixed in 2% PFA + 0.2% glutaraldehyde in 0.1 M PHEM buffer for 10 min, deyolked and fixed for an additional 2 h in the same solution. Cells of interest in *red* (primordial germ cells [PGCs] in this example, marked with *asterisk*). **(B)** Samples were embedded in 4% low melting point (LMP) agarose within a plastic mold. **(C)** LMP-agarose block was sectioned into 100-μm-thick slices. Slices were examined with a fluorescent microscope and those containing cells of interest were collected. **(D)** The slices were glued with a drop of LMP-agarose onto glass or carbon-coated gridded coverslips. **(E)** Laser marking, for example, in shape of an *arrowhead* was introduced using an 850-nm pulsed laser on a two-photon setup (in 0.1 M PHEM buffer). **(F)** Strong fixation in 2% PFA + 2% glutaraldehyde for 2 h and transfer to 1% PFA in 0.1 M PHEM solution. **(G)** Post-fixation with 1% osmium tetroxide, followed by embedding in EPON. **(H)** *Arrow* relocation using light microscope and trimming the EPON. **(I)** Sectioning 200-nm-thick slices at 5 μm intervals until reaching the laser mark. **(J)** Ultrathin (60 nm) sectioning at laser-marked location. **(K)** Transmission electron microscopy. Color images available online at www.liebertpub.com/zeb

Generation of a laser mark using an 850-nm pulsed laser on a two-photon setup (We used 100% power and 1000–2500 iterations. Power and iterations can be adjusted based on depth of cells and laser power output) (Fig. 1E). To prevent sample photo bleaching, we used the ''test bleach'' mode in ZEN software, equivalents can be used in other setups.

Performing confocal imaging of the cells for three-dimensional reconstruction (e.g., using a 63× objective with NA: 1.0, 0.8 μm z-slice and pinhole 1 AU) (Fig. 1E and Supplementary Movie S2).

For an overview of the samples in lower magnification, capture a photo using 5× and 20× objectives (the 20× objective is used for measurement of X/Y/Z coordinates of the cells). Depth of the cells (Z) from surface of the tissue was measured by subtracting the microscope stage position while focusing on the cells from that of the stage position when focusing on the tissue surface.

Strong fixation with 2% PFA + 2% glutaraldehyde in 0.1 M PHEM at room temperature for 2 h under the fume hood. Continue by exchanging solution with 1% PFA in the 0.1 M PHEM buffer at 4°C (Fig. 1F). Continue to the next step as soon as possible, as prolonged storage can degrade the quality of the sample.

Postfixing in 1% osmium tetroxide, containing 1.5% potassium cyanoferrate, and embedding in EPON after dehydration[10] (Fig. 1G).

Relocating the laser mark in EPON by illuminating the block under the microscope in brightfield and trimming the block to the area containing the laser mark (keeping the sample in the transmission electron microscopy (TEM) sample holder helps to avoid altering the position or the orientation of the sample) (Fig. 1H and Supplementary Movie S3).

Targeted sectioning by generating 200-nm-thick slices at 5 μm intervals and examining them for the presence of the laser mark (Fig. 1I).

Ultrathin sectioning (60 nm) at position of the laser mark (Fig. 1J) and TEM imaging (Fig. 1K).

Compare the light microscope and TEM images to identify the cells of interest (Supplementary Fig. S1).

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dagmar Zeuschner, PhD
Electron Microscopy Unit
Max Planck Institute for Molecular Biomedicine
Röntgenstrasse 20
Münster D-48149
Germany

E-mail: d.zeuschner@mpi-muenster.mpg.de

Erez Raz, PhD
Institute of Cell Biology
ZMBE
University of Münster
Von-Esmarch str. 56
Münster 48149
Germany

E-mail: erezraz@uni-muenster.de