

Oct 28, 2021

# Immuno-correlative light and electron microscopy (iCLEM) using STEM

Viola Oorschot<sup>1</sup>, Jillian C Danne<sup>1</sup>, Benjamin Lindsey<sup>2</sup>, Jan Kaslin<sup>2</sup>,  
Georg Ramm<sup>1</sup>

<sup>1</sup>Ramaciotti Centre for Cryo EM, Monash University, Melbourne, Australia;

<sup>2</sup>Australian Regenerative Medicine Institute, Monash University, Melbourne Australia

1



[dx.doi.org/10.17504/protocols.io.bv89n9z6](https://dx.doi.org/10.17504/protocols.io.bv89n9z6)



Jillian Danne

*Immuno-*correlative light and electron microscopy (iCLEM) combines ultrastructural information obtained from high resolution electron microscopy and the use of genetically encoded or cytochemical markers. *Immuno*-CLEM takes advantage of the antigenicity preserved by Tokuyasu sample preparation to identify, quantify and characterise heterogeneous cell populations in small organisms, organs and tissue of healthy and diseased states. iCLEM can be used in combination with scanning EM (SEM), scanning TEM (STEM), and transmission EM (TEM). These protocols are well-suited, for example, for investigating neural stem and progenitor cell populations of the vertebrate nerve system and are available as separate protocols on protocol.io. Here, a method for iCLEM-STEM is described using an adult zebrafish telencephalon brain as a model. This organ is small in size allowing the complete dorsal telencephalic niche to be visualised in sections, and has diverse cell profiles and regenerative potential of local neural stem and progenitor cells. iCLEM-STEM involves the examination of ultrathin tissue sections (62-70 nm) using immunofluorescence labelling and subsequent SEM imaging to obtain a high resolution overview of these sections with greater morphological detail compared to iCLEM-SEM. This protocol should be of particular interest to EM facilities with SEM, but not TEM access.

Oorschot et al. 2021. TEM, SEM, and STEM-based immuno-CLEM workflows offer complimentary advantages.pdf  
Slot and Geuze 2007 Cryosectioning\_and\_Immuno-labeling.pdf

DOI

[dx.doi.org/10.17504/protocols.io.bv89n9z6](https://dx.doi.org/10.17504/protocols.io.bv89n9z6)

<https://store.erc.monash.edu.au/experiment/view/14488/>

Viola Oorschot, Jillian C Danne, Benjamin Lindsey, Jan Kaslin, Georg Ramm  
2021. Immuno-correlative light and electron microscopy (iCLEM) using STEM.  
**protocols.io**  
<https://dx.doi.org/10.17504/protocols.io.bv89n9z6>



protocol

Oorschot, Viola, et al. "TEM, SEM, and STEM-based immuno-CLEM workflows offer complementary advantages." Scientific reports 11.1 (2021): 1-16.

Correlative light and electron microscopy, Tokuyasu technique, Transmission electron microscopy, Zebrafish, Fluorescence microscopy, Stem cells, Progenitor cells, Scanning electron microscopy

protocol ,

Oorschot, Viola, et al. "TEM, SEM, and STEM-based immuno-CLEM workflows offer complementary advantages." Scientific reports 11.1 (2021): 1-16.

Jun 30, 2021

Oct 28, 2021

51201

[Paraformaldehyde, 16% \(wt/vol\)](#) **Electron Microscopy**

**Sciences Catalog #15710**

[Glutaraldehyde 8% \(wt/vol\)](#) **Polysciences**

**Inc Catalog #00216**

[Sucrose](#) **Merck**

**Millipore Catalog #1.07654.1000**

[Formvar powder](#)

**ProSciTech Catalog #C064**

[0.1M Phosphate buffer pH 7.4](#) **Contributed by users**


[0.2M Phosphate buffer pH 7.4](#) **Contributed by users**

[1x Phosphate buffered saline pH 7.4](#) **Contributed by users**

[☒ Glycine Sigma](#)  
**Aldrich Catalog #G-7403**  
[☒ Gelatin from porcine skin Sigma](#)  
**Aldrich Catalog #G1890-500g**  
[☒ Methyl cellulose Sigma](#)  
**Aldrich Catalog #M-6385**  
[☒ Methylene blue Merck Millipore](#)  
**Sigma Catalog #115943**  
[☒ Azure II Merck Millipore](#)  
**Sigma Catalog #861065**  
[☒ MilliQ water Contributed by users](#)  
[☒ Toluidine blue Merck Millipore Sigma](#)  
[☒ Bovine serum albumin, heat shock fraction pH7 Sigma](#)  
**Aldrich Catalog #A9647**  
[☒ Mouse anti-glutamine synthetase antibody Merck](#)  
**Millipore Catalog #MAB302**  
[☒ Biotinylated anti-eGFP](#)  
**antibody Rockland Catalog #600-106-215**  
[☒ Rabbit anti-biotin](#)  
**antibody Rockland Catalog #100-4198**  
[☒ Goat anti-mouse alexaFluor-555](#)  
**antibody Thermofisher Catalog #A21424**  
[☒ Goat anti-rabbit alexaFluor-488](#)  
**antibody Thermofisher Catalog #A11008**  
[☒ Rabbit anti-mouse Ig](#)  
**antibody Rockland Catalog #610-4120**  
[☒ Protein A-Gold 10nm UMC](#)  
**Utrecht Catalog #PAG 10nm**  
[☒ Protein A-Gold 20nm UMC](#)  
**Utrecht Catalog #PAG 20nm**  
[☒ Hoechst 33342 Thermo Fisher](#)  
**Scientific Catalog #62249**  
[☒ 2% Uranyl oxalate pH 7.0 Contributed by users](#)  
[☒ Uranyl acetate Electron Microscopy](#)  
**Sciences Catalog #22400**

 [Liquid nitrogen](#) Contributed by users

 [Ice](#) Contributed by users

 [Glycerol >99% Sigma](#)

**Aldrich Catalog #G5516**

Double edge blades

Personna      72000

Black teflon plate

N/A      N/A

Fine Forceps

Forceps

Dumont      11251-10      



Blade scalpel ST #11

Swann Morton      21016SM

Bite and boxing wax-500g

Investo (Lordell)      WI-BB

Micro spatula, stainless steel narrow spoon

ProSciTech      T1453

Dry block heater

Ratek      DBH4000D

Sample pin for cryo-ultramicrotomes, aluminium

Leica      75959-06

Embedding mould, single ended flat 21 cavities

ProSciTech      RL064

Falcon® Centrifuge Tubes  
Polypropylene, Sterile, 15 mL

Corning®      352096

Falcon® Centrifuge Tubes  
Polypropylene, Sterile, 50 mL  
Corning® 352070

Tube 5ml 5016 PP yellow cap GS  
Pacific Laboratory Products P5016SU

UC7/FC7 Cryo-ultramicrotome  
Leica EMFC7

Cryotrim20 diamond knife  
Diatome TT-20

Cryo immuno diamond knife, 3mm  
Diatome DCIMM3530

Perfect loop  
Diatome 70944

Mini hot plate

Thermofisher      HP2310BQ

50 Mesh copper grids

Gilder      GCU50

50 Mesh palladium/copper grids

Gilder      GCU-PD50

150 Mesh copper grids

Gilder      GCU150

150 Mesh palladium/copper grids

Gilder      GCU-PD150

Stainless steel loop, 3mm

Contributed by user      N/A

Remanium wire loop

N/A

N/A

Plain glass slides 76mm x 39mm x 1.0-1.2mm

Thermo Scientific

AGL4222A

Leica AF6000LX widefield microscope, with 63x 1.3NA glycerol objective

Leica

N/A

Olympus widefield microscope, model CHK2-F-GS

Olympus

N/A

Petri dish 100mm x 20mm

Greiner Bio-One

664160

24-well clear flat bottom TC-treated multi-well cell culture plate

Falcon

FAL353047



Transfer pipette, standard bulb, PE, fine tip, capacity 5ml

ProSciTech      LCH192

Parafilm M

Bemis      IA041

Glass board

N/A      N/A

Glass petri dish, 100mm

BRAND      BR455751

Oven MINO/6/CLAD

Genlab      N/A

Coverslip 22mm x 22mm, No 1

Menzel Glaser      CS22221G

30mm Glass bottom culture dish

ProSciTech      H500730

Filter paper, grade 1, 12.5cm

Whatman      1001-125

Polystyrene esky

N/A      N/A

Leica EM ACE200 coater

Leica      N/A

JEOL JEM-1400 Plus 120keV  
Transmission electron microscope

JEOL      N/A

Liquid Nitrogen is extremely cold (-196 degrees Celsius) and can cause severe burns if not handled properly. Personal Protective Equipment (PPE) must be worn when handling liquid nitrogen.

Uranyl Acetate is radioactive and acutely toxic. Personal protective equipment must

be worn when handling this substance.

Paraformaldehyde and glutaraldehyde are toxic, corrosive and potentially carcinogenic. These chemicals must be handled in a fume hood using the appropriate PPE.

### Tissue fixation

- 1 Dissect out the tissue of interest (example, forebrain of Tg(proliferating cell nuclear antigen:GFP) transgenic adult zebrafish with olfactory bulbs attached for tissue orientation) on a teflon plate or dental wax sheet using fine forceps and a scalpel blade, at room temperature and place in fixative, either 2% paraformaldehyde, 0.2% glutaraldehyde in 0.1M phosphate buffer (PB) (pH 7.4) or 4% paraformaldehyde in 0.1M PB (pH 7.4). Keep the tissue submerged in fixative at all times.

Always perform fixation using a fume hood and wear appropriate personal protective equipment (PPE).

- 2 Place the tissue in 5 ml tubes containing the fixative used in Step 1 (2% paraformaldehyde, 0.2% glutaraldehyde in 0.1M PB (pH 7.4) or 4% paraformaldehyde in 0.1M PB (pH 7.4)) and fix overnight, at 4 degrees Celsius on a rotor. For brain samples, add 4% sucrose to the fixation solution.
- 3 Samples can be stored in 1% paraformaldehyde in 0.1M PB at 4 degrees Celsius until further processing.

### Tokuyasu embedding and sectioning

- 4 Remove the fixative and wash with phosphate buffered saline (PBS), 3 x 10 mins.
- 5 Wash with 0.15% glycine in PBS for 10 mins.
- 6 Infuse tissue with pre-warmed 6% gelatin in 0.1M PB for 1 hour at 37 degrees Celsius, agitating. Use a heat block to maintain the temperature.
- 7 Infuse tissue again with pre-warmed 6% gelatin in 0.1M PB for 1 hour at 37 degrees Celsius, agitating.

- 8 Place gelatin infused tissue in plastic embedding moulds containing pre-warmed 6% gelatin in 0.1M PB at 37 degrees Celsius, and solidify gelatin blocks by cooling at 4 degrees Celsius for 60 mins.

Alternatively, the flat embedding method can be used to minimise gelatin block shrinkage following sucrose infiltration. Add 6 percent pre-warmed gelatin in 0.1M PB to a petri dish and solidify at 4 degrees Celsius for 1 hour. Place the tissue on top in a layer of 6 percent gelatin pre-warmed to 37 degrees Celsius. Solidify gelatin at 4 degrees Celsius for 1 hour.

- 9 Place the gelatin infused tissue blocks on a teflon plate or dental wax sheet and cut off excess gelatin from around the tissue using a razor blade or scalpel.

For flat embedded samples, cut around the tissue using a razor blade or scalpel and remove the gelatin embedded sample from the petri dish using a small spatula. Cut off excess gelatin using a razor blade or scalpel.

- 10 Fix the gelatin infused tissue blocks in 0.2% paraformaldehyde in 0.1M PB for 30 mins at 4 degrees Celsius, rotating.

- 11 Wash tissue blocks with 0.1M PB, 3 x 10 mins, agitating.

- 12 Infuse tissue blocks with 2.3M sucrose in 0.1M PB for 2 days at 4 degrees Celsius, rotating.

- 13 Mount each block on a clean aluminium bullseye pin. Position tissue in the correct orientation and remove the excess sucrose with strips of filter paper.

- 14 Freeze each sample block by gently submerging the pins in liquid nitrogen. For large blocks, cool the sample pin in the chamber of a cryo-ultramicrotome (-100 degrees Celsius) for 10 mins prior to freezing in liquid nitrogen. Pins should be mounted and frozen one at a time.

- 15 Trim the front face and edges of the frozen tissue block cutting 50-100 µm deep on all four sides. Cut at a speed of 100 mm/sec with a 100 nm feed, at -90 to -100 degrees Celsius using a Leica UC7/FC7 cryo-ultramicrotome and Diatome cryotrim 20 knife.

- 16 To check the region of interest:

- 16.1 Cut semi thin sections with a feed of 100-300 nm, at 3 mm/sec and at -90 to -100 degrees Celsius.
  - 16.2 Pick up sections in a 1:1 mixture of 2% methylcellulose: 2.3M sucrose in 0.1M PB using a 3 mm stainless steel loop.
  - 16.3 Place sections on a slide and stain with methylene blue/Azure II or toluidine blue solution for 10-20 seconds before rinsing with water and drying on a mini hotplate.
  - 16.4 Observe sections with a wide-field light microscope. If the region of interest has not been obtained, continue trimming and repeat Step 16.
- 17 Once the region of interest has been obtained, cut 62-70 nm ultrathin sections at 0.8 mm/sec and -90 to -100 degrees Celsius using a Leica UC7/FC7 cryo-ultramicrotome and Diatome cryo immuno knife.
  - 18 Pick up ultrathin sections in a 1:1 mixture of 2% methylcellulose : 2.3M sucrose in 0.1M PB using a 3 mm stainless steel loop, and place sections on 50-150 mesh copper or palladium coated copper grids with carbon-coated formvar film.  
  
Store grids in an enclosed petri dish at 4 degrees Celsius until use.
  - 19 62-70nm ultrathin sections can be used for immunofluorescence (IF) labelling for optical microscopy using different cellular markers (Steps 20-37) and then subsequent SEM imaging to obtain a high resolution overview (Steps 38-48).

#### Immunofluorescent labelling of ultrathin sections for optical microscopy using two cellular markers

- 20 Fill the wells of a 24-well plate with PBS and float grids section side down.
- 21 Use a fine tip pasteur pipette to remove enough PBS from each well so that the lid can be secured on the well-plate without disturbing the floating grids. Seal the lid with parafilm.
- 22 Put the well-plate containing the grids in an oven set to 50-60 degrees Celsius for 1 hour to remove the 2% methylcellulose : 2.3M sucrose and 6% gelatin from the tissue sections.

- 23 Place a sheet of parafilm on a clean flat bench or a glass board using a small amount of water underneath to keep the film flat.

For the following steps, use a perfect loop or fine forceps to transfer grids from one drop of solution to another, section side down. Use approximately 100-200  $\mu$ l drops for rinsing solutions and 5  $\mu$ l drops for antibodies on a clear parafilm surface. Centrifuge all antibodies for 30 seconds using a benchtop microcentrifuge prior to use.

- 24 Quench aldehydes with 0.15% glycine in PBS, 5 x 2 mins.

- 25 Block with 1% bovine serum albumin (BSA) in PBS for 5 mins.

- 26 Incubate with both primary antibodies diluted in 1% BSA/PBS for 45 to 60 mins in a dark moist chamber, at room temperature.

Example: Mouse anti-glutamine synthetase (1:500 dilution), to label glial cells.

Example: Biotinylated anti-eGFP (1:300 dilution), to label green fluorescent protein labelled proliferating cell nuclear antigen (PCNA).

- 27 Rinse with 0.1% BSA in PBS, 5 x 2 mins.

- 28 Optionally incubate with a bridging antibody diluted in 1% BSA/PBS for 30 mins at room temperature.

Example: Rabbit anti-biotin (1:10,000 dilution)

- 29 Rinse with 0.1% BSA in PBS, 5 x 2 mins.

- 30 Incubate with both secondary antibodies diluted in 1% BSA/PBS for 45 mins in a dark moist chamber, at room temperature.

Example: Goat anti-mouse AlexaFluor-555 (1:300 dilution).

Example: Goat anti- rabbit AlexaFluor-488 (1:300 dilution)

- 31 Rinse with PBS, 5 x 2 mins.
- 32 Rinse with distilled water, 4 x 2 mins.
- 33 Incubate with Hoechst nuclear stain (1  $\mu$ M in distilled water) for 20 mins.
- 34 Rinse with water, 4 x 2 mins.

#### Fluorescent optical microscopy imaging for grids

- 35 Place grid section side up in a drop of 50% glycerol in water. Ensure the grid is completely covered in glycerol with no air bubbles present.
- 36 Place grid section side down on a 30 mm glass bottom culture dish and cover grid with a glass coverslip.
- 37 Acquire a fluorescent montaged z-stack of 62-70 nm ultrathin tissue sections using an inverted widefield fluorescent microscope.

Example: Leica AF6000LX with a DFC 350FX camera, and a 40x 0.6 NA dry or 63x 1.3 NA glycerol objective.

After imaging, prepare the grids for scanning electron microscopy as follows:

#### Preparation of immunofluorescence grids for scanning transmission electron microscopy

- 38 Add distilled water to the 30 mm glass bottom culture dish and agitate gently until the coverslip floats.

- 39 Remove the coverslip and lift the grid out using fine forceps.
- 40 Carefully remove water from the backside of the grid by placing filter paper at the grid edge.
- 41 Put a drop of distilled water on a sheet of parafilm and place the grid on the drop. The grid should float.
- 42 Rinse with distilled water, 6 x 2 mins. Water drops should be placed on a clean parafilm sheet.
- 43 Stain the grid in a drop of 2% Uranyloxalate (pH 7.0) for 5 mins at room temperature.
- 44 Rinse once with distilled water.
- 45 Cover a glass petri dish with parafilm using a small amount of water under the film to keep it flat. Place the dish on ice and add 3 drops of filtered 2% methylcellulose : 4% uranyl acetate (9 ml : 1 ml) (pH 4.0) to the surface.
- 46 Float the grid briefly in the first two drops of methylcellulose/uranyl acetate, then transfer to the third drop and leave for 10 minutes.
- 47 Remove and dry the grid following the looping out method:
  - 47.1 Clean a rermanium wire loop with water and dry.
  - 47.2 Push the loop into the the methylcellulose/uranyl acetate drop and under the



grid.

- 47.3 Lift the grid out from the drop using the loop.
  - 47.4 Tilt the loop and grid at a 45-60 degree angle and bring the loop to a piece of filter paper.
  - 47.5 As soon as the loop contacts the filter paper, drag it slowly along the filter paper to remove excess liquid. A thin film of methylcellulose-uranyl acetate should remain on the surface of the grid. The slower the drag, the thinner the film.
  - 47.6 Dry the grid in the loop for 30 minutes at room temperature.
  - 47.7 Remove the grid from the loop using fine forceps, taking care not to tear the methylcellulose/uranyl acetate film away from the grid. Store the grid in a grid box.
- 48 Place the grid in a specialised STEM grid holder for image acquisition of a high resolution overview using a scanning electron microscope.

Example: Thermofisher/FEI Nova NanoSEM 450 equipped with a bottom mount STEM II (HAADF) detector set at 30 keV with a working distance of 6.8 mm, dwell time of 3  $\mu$ s, and with MAPS 2.0 software used for generating montages and correlation.