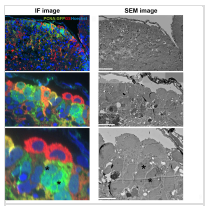

Oct 28, 2021

Immuno-correlative light and electron microscopy (iCLEM) using SEM

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Jillian Danne

Immuno- correlative light and electron microscopy (*i*CLEM) combines ultrastructural information obtained from high resolution electron microscopy with 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. *i*CLEM 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 *i*CLEM-SEM 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. *i*CLEM-SEM provides a large quantifiable overview of 200 nm tissue sections without the presence of grid bars, and thicker sections enhance the immunofluorescent labelling.

Oorschot et al. 2021. TEM, SEM, and STEM-based immuno-CLEM workflows offer complimentary advantages.pdf

Slot and Geuze 2007 Cryosectioning_and_Immunolabeling.pdf

DOI

dx.doi.org/10.17504/protocols.io.bu54ny8w

http://Example data: 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 SEM. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bu54ny8w

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, 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.

May 19, 2021

Oct 28, 2021

50076

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**

Sodium cacodylate trihydrate **Merck Millipore Sigma Catalog #C0250**

Osmium tetroxide 1g **ProSciTech Catalog #C010-1010**

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Potassium ferricyanide (III) **Merck Millipore Sigma Catalog #702587**

Tannic acid **Merck Millipore Sigma Catalog #W304204**

2% Uranyloxylate pH 7.0 **Contributed by users**

Uranyl acetate **Electron Microscopy Sciences Catalog #22400**

Liquid nitrogen **Contributed by users**

Ice **Contributed by users**

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**

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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**

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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**

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Perfect loop

Diatome **70944**

Mini hot plate

Thermofisher **HP2310BQ**

Stainless steel loop, 3mm

Contributed by user **N/A**

Remanium wire loop

N/A **N/A**

u-Slide 8 well high ibiTreat #1.5 polymer coverslip, tissue culture treated, sterlised

Ibidi **80806**

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

Thermo Scientific **AGL4222A**

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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**

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

ProSciTech **LCH192**

Parafilm M

Bemis **IA041**

Glass petri dish, 100mm

BRAND **BR455751**

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Oven MINO/6/CLAD

Genlab **N/A**

Coverslip 22mm x 22mm, No 1

Menzel Glaser **CS22221G**

Filter paper, grade 1, 12.5cm

Whatman **1001-125**

3.5mm Rapid core biopsy punch

ProSciTech **T983-35**

Polystyrene esky

N/A **N/A**

Leica EM ACE200 coater

Leica **N/A**

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FEI Nova Nano Scanning electron microscope 450

Thermofisher **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.

Osmium tetroxide is corrosive, toxic and an irritant. This chemical must be handled in a fume hood using the appropriate PPE.

Sodium cacodylate buffer contains arsenic and is acutely toxic. This chemical 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

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processing.

Tokuyasu embedding and sectioning

4 Remove 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 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.

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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 200 nm semithin sections at 3 mm/sec and -90 to -100 degrees Celsius using a Leica UC7/FC7 cryo-ultramicrotome and Diatome cryo immuno knife.

18 Pick up semithin 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 directly in a carbon-coated µ-slide 8 well

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high microscope Ibidi chamber with a #1.5 polymer coverslip surface.

Store sections in a sealed microscope Ibidi chamber at 4 degrees Celsius until use.

19 200 nm semi thin sections can be used for immunofluorescence (IF) labelling for optical microscopy using different cellular markers (Steps 20-33) and then subsequent SEM imaging to obtain a quantifiable overview of a region of interest (Steps 34-49).

Immunofluorescent labelling for optical microscopy using two cellular markers

20 Perform the following steps directly in the wells of an Ibidi chamber. Use approximately 300 µl for rinsing solutions and 20 µl for antibodies. Centrifuge all antibodies for 30 seconds using a benchtop microcentrifuge prior to use.

Wash with PBS and place the ibidi chamber at 50-60 degrees Celsius for 1 hour to remove the 2% methylcellulose : 2.3M sucrose and 6% gelatin from the tissue sections.

21 Wash with PBS, 5 x 2 mins at room temperature.

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

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

24 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).

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

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

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26 temperature.

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

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

28 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)

29 Rinse with PBS, 5 x 2 mins.

30 Rinse with distilled water, 4 x 2 mins.

31 Incubate with Hoechst nuclear stain (1µM in distilled water) for 20 mins.

32 Rinse with water, 4 x 2 mins.

Fluorescent optical microscopy imaging for ibidi chambers

33 Acquire a fluorescent montaged z-stack of 200 nm semithin sections in distilled water using an inverted widefield 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 semithin sections for scanning electron microscopy as follows:

Preparation of sections for scanning electron microscopy

34 Perform the following steps directly in Ibidi chambers and in the fume hood using the appropriate PPE:

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Fix 200 nm sections with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) overnight at 4 degrees Celsius.

35 Rinse with 0.1M sodium cacodylate buffer (pH 7.4), 3x 5 mins at room temperature.

36 Post fix with 2% osmium tetroxide and 1.5% potassium ferricyanide in 0.1M sodium cacodylate buffer for 90 mins at 4 degrees Celsius.

37 Rinse with 0.1M sodium cacodylate buffer, 6 x 5 mins at room temperature.

38 Fix with 1% Tannic acid in 0.1M sodium cacodylate buffer for 30 mins at room temperature.

39 Rinse with 0.1M sodium cacodylate buffer, 6 x 5 mins.

40 Incubate sections in 1% osmium tetroxide in distilled water for 30 mins at 4 degrees Celsius.

41 Rinse with distilled water, 6 x 5 mins.

42 Punch out the tissue section with the polymer coverslip from the Ibidi chamber using a rapid-core 3.5 mm biopsy punch. Handle the polymer coverslip with the tissue section using tweezers or a perfect loop as an EM grid would be handled.

43 Transfer the section to a drop of 2% Uranyloxalate (pH 7.0), and stain for 5 mins at room temperature.

44 Rinse once with distilled water.

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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 coverslip with section 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 section following the looping out method:

47.1 Clean a remanium wire loop with water and dry.

47.2 Push the loop into the the methylcellulose/uranyl acetate drop and under the coverslip/section.

47.3 Lift the coverslip/section 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 section. The slower the drag, the thinner the film.

47.6 Dry the section in the loop for 30 minutes at room temperature.

47.7 Remove the coverslip/section from the loop using fine forceps, taking care not to tear the methylcellulose/uranyl acetate film away from the section. Mount the coverslip/section on an SEM stub using a carbon tab section side up.

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48 Coat the stub and mounted section with 2 nm of iridium or platinum.

49 Mount the stub on a standard SEM holder for imaging using a scanning electron microscope.

Example: Thermofisher/FEI Nova NanoSEM 450 equipped with a retractable back scatter electron detector set at 10 keV with a working distance of 5 mm, dwell time of 3 µs, and with MAPS 2.0 software used for generating montages and correlation.

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