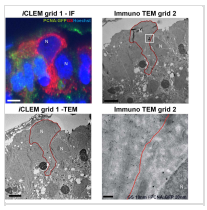

Oct 28, 2021

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

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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-TEM 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-TEM provides high resolution ultrastructural detail of cells, and consecutive ultrathin (62-70 nm) tissue sections can be examined using different labelling techniques involving the use of immunofluorescent and immunogold markers.

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.btmrnk56

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 TEM. **protocols.io** https://dx.doi.org/10.17504/protocols.io.btmrnk56

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, Immunogold labelling

protocol ,

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

Mar 24, 2021

Oct 28, 2021

48529

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**

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2% Uranyloxylate 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

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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**

Falcon® Centrifuge Tubes

Polypropylene, Sterile, 15 mL

Corning® **352096**

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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**

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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**

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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

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

ProSciTech **LCH192**

Parafilm M

Bemis **IA041**

Glass board

N/A **N/A**

Sprout plus mini centrifuge

Sprout **120610**

Oven MINO/6/CLAD

Genlab **N/A**

Coverslip 22mm x 22mm, No 1

Menzel Glaser **CS22221G**

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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

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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 the 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.

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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 solidified 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:

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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 milliQ 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 Consecutive (serial) 62-70 nm tissue sections of the same cell type can be used for the following workflows:

1. Immunofluorescence (IF) labelling of ultrathin sections for optical microscopy using different cellular markers (Steps 20-37) and then preparation of these grids for subsequent TEM imaging to obtain ultrastructural detail (Steps 38-48).

2. Single or double-immunogold labelling of ultrathin sections and TEM imaging to validate the specificity of IF markers at high resolution (Steps 49-77).

When used in combination, these methods allow for the correlation of two fluorescent markers with two different sized protein-A-gold markers within the same cell, as described below.

Immunofluorescent labelling for optical microscopy using two cellular markers

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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 µl drops for rinsing solutions and 5 µl 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.

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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 µM 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.

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37 Acquire a fluorescent montaged z-stack of 62-70 nm ultrathin 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 transmission electron microscopy as follows:

Preparation of immunofluorescence grids for 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

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the third drop and leave for 10 minutes.

47 Remove and dry the grid following the looping out method:

47.1 Clean a remanium wire loop with water and dry.

47.2 Push the loop into 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 TEM grid holder for high resolution imaging using a transmission electron microscope.

Example: JEOL 1400-Plus 120 keV TEM equipped with a high sensitivity bottom mount CMOS 'Flash' camera.

Immunogold double-labelling of ultrathin sections for transmission electron microscopy

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49 Fill the wells of a 24-well plate with PBS and float grids section side down in the solution. 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. Secure the plate lid with parafilm.

50 Put the plate with the floating grids in an oven set to 50-60 degrees Celsius for 60 minutes to remove the 2% methylcellulose/2.3M sucrose and 6% gelatin from the tissue sections.

51 Place a sheet of parafilm on the bench 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 µl drops for rinsing and 5 µl drops for antibodies on a clean parafilm surface. Spin all antibodies and protein A-gold (PAG) for 30 seconds using a benchtop microcentrifuge prior to use.

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

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

54 Incubate with a primary antibody diluted in 1% BSA/PBS for 45 to 60 minutes in a dark moist chamber at room temperature.

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

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

56 Optionally incubate with a bridging antibody diluted in 1% BSA/PBS for 20 mins, in a dark moist chamber, at room temperature.

Example: Rabbit anti-mouse Ig bridging antibody (1:1000 dilution).

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

Incubate with 10 nm PAG (dilution as recommended by manufacturer for specific batch)

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58 diluted in 1% BSA/PBS for 30 mins, in a dark moist chamber, at room temperature.

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

60 Rinse with PBS, 5 x 2 mins.

61 Fix with 1% glutaraldehyde in PB for 5 mins at room temperature to prevent non-specific binding of subsequent PAG particles and to stabilise the immunogold labelling reaction. This step must be completed in the fume hood using appropriate PPE.

62 Rinse with PBS, 2 x 5 mins.

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

Example: Biotinylated enhanced green fluorescent protein (eGFP) (1:500 dilution).

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

65 Optionally incubate with a bridging antibody (1:10,000 dilution) diluted in 1% BSA/PBS for 30 mins in a dark moist chamber at room temperature.

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

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

67 Incubate with 20 nm PAG (dilution as recommended by manufacturer for specific batch) diluted in 1% BSA/PBS for 30 mins in a dark moist chamber, at room temperature.

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

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69 Rinse with PBS, 5 x 2 mins.

70 Fix in 1% glutaraldehyde in PB for 5 mins at room temperature to stabilise the immunogold-labelling reaction. This step must be completed in the fume hood using appropriate PPE.

71 Rinse with PBS, 2 x 5 mins.

72 Rinse with distilled water, 6 x 1 min.

73 Stain the grid in a drop of 2% Uranyloxalate (pH 7.0) for 5 mins at room temperature.

74 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 2% methylcellulose : 4% uranyl acetate (9 ml : 1 ml) (pH 4.0) to the surface.

75 Float the grid briefly in the first two drops of methylcellulose/uranyl acetate (pH 4.0), then transfer to the third drop and leave for 10 mins.

76 Remove and dry the grid by the looping out method, described above (Step 47).

77 Acquire high resolution micrographs using a transmission electron microscope.

Example: JEOL-1400 Plus 120 keV TEM equipped with a high sensitivity bottom mount CMOS 'Flash' camera.

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