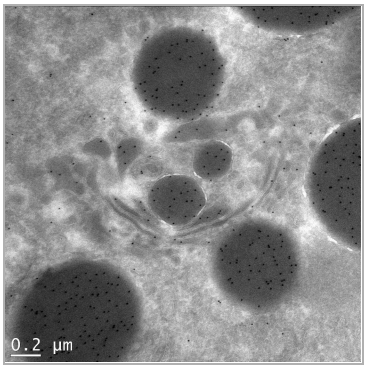

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Tokuyasu processing and immuno-electron microscopy of tissue

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ABSTRACT

Ultrastructural morphological information is routinely obtained from the processing of cells and tissue into plastics for transmission electron microscopy imaging. This technique involves the use of strong chemical fixatives, solvents and resins, which destroy the antigenicity of samples. Freezing samples to a vitreous state using cryo-electron microscopy techniques preserves ultrastructure without the use of harsh chemicals, however, immunolabelling cannot be achieved at temperatures as low as -196 degrees Celsius. Here we outline a protocol for the Tokuyasu technique for cryopreservation and immunolabelling of subcellular structures in tissue. Tissue is lightly chemically fixed and cryoprotected in sucrose before freezing in liquid nitrogen. Tissue samples are sectioned by cryo-ultramicrotomy, and retrieved cryosections are thawed, immunolabelled, stained and imaged using a transmission electron microscope.

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Protocol status: Working
We use this protocol and it's working

Created: Oct 31, 2022

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PROTOCOL integer ID: 72104

MATERIALS

- Paraformaldehyde, 16% (wt/vol) **Electron Microscopy Sciences Catalog #15710**
- Glutaraldehyde 8% (wt/vol) **Polysciences, Inc. Catalog #00216**
- 0.1M Phosphate buffer pH 7.4 **Contributed by users**
- 0.2M Phosphate buffer pH 7.4 **Contributed by users**
- Sucrose **Merck Millipore (EMD Millipore) Catalog #1.07654.1000**

Keywords: Tokuyasu, Protein A Gold, Cryo-ultramicrotomy, Transmission Electron Microscope, Immunogold labelling, Vitrification, Ultrastructure, Tissue, Antibodies

- 1x Phosphate buffered saline pH 7.4 **Contributed by users**
- Glycine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G-7403**
- Gelatin from porcine skin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G189 500g**
- Ice **Contributed by users Catalog #N/A**
- Liquid nitrogen **Contributed by users**
- Methyl cellulose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M-6385**
- MilliQ water **Contributed by users**
- Formvar powder **ProSciTech Catalog #C064**
- Azure II **Merck MilliporeSigma (Sigma-Aldrich) Catalog #861065**
- Methylene blue **Merck MilliporeSigma (Sigma-Aldrich) Catalog #115943**
- Toluidine blue **Merck MilliporeSigma (Sigma-Aldrich) Catalog #89640-5G**
- Bovine serum albumin heat shock fraction pH 7 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9647**
- Mouse anti-glutamine synthetase antibody **Merck Millipore (EMD Millipore) Catalog #MAB302**
- Rabbit anti-mouse Ig antibody **Rockland Catalog #610-4120**
- Protein A-Gold 10nm **UMC Utrecht Catalog #PAG 10nm**
- 2% Uranyloxylate pH 7.0 **Contributed by users**
- Uranyl acetate **Electron Microscopy Sciences Catalog #22400**

Equipment

NAME	
Black teflon plate	
BRAND	N/A
SKU	N/A

Equipment

Bite and boxing wax-500g NAME

Investo (Lordell) BRAND

WI-BB SKU

Equipment

Fine Forceps NAME

Dumont BRAND

11251-10 SKU

Equipment

Blade scalpel ST #11 NAME

Swann Morton BRAND

21016SM SKU

Equipment

Sagittal Matrix, Mouse, 30g Adult, 1mm NAME

Pelco® BRAND

15004 SKU

Equipment

Rotary tube mixer NAME

Ratek Instruments BRAND

RSM7DC SKU

Equipment

Parafilm M NAME

Bemis BRAND

IA041 SKU

Equipment

Tube 5ml 5016 PP yellow cap GS NAME

Pacific Laboratory Products BRAND

P5016SU SKU

Equipment

Dry block heater NAME

Ratek BRAND

DBH4000D SKU

Equipment

BRAND® Petri dish, glass 150mm x 25mm

NAME

BRAND®

BRAND

BR455751

SKU

Equipment

Petri dish 100mm x 20mm

NAME

Greiner Bio-One

BRAND

664160

SKU

Equipment

Double edge blades

NAME

Personna

BRAND

72000

SKU

Equipment

Micro spatula, stainless steel narrow spoon

NAME

ProSciTech

BRAND

T1453

SKU

Equipment

Polystyrene esky

NAME

N/A

BRAND

N/A

SKU

Equipment

Sample pin for cryo-ultramicrotomes, aluminium

NAME

Leica

BRAND

75959-06

SKU

Equipment

Filter paper, grade 1, 12.5cm

NAME

Whatman

BRAND

1001-125

SKU

Equipment

UC7/FC7 Cryo-ultramicrotome

NAME

Leica

BRAND

EMFC7

SKU

Equipment

Cryotrim20 diamond knife

NAME

Diatome

BRAND

TT-20

SKU

Equipment

Stainless steel loop, 3mm

NAME

Contributed by user

BRAND

N/A

SKU

Equipment

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

NAME

Thermo Scientific

BRAND

AGL4222A

SKU

Equipment

Mini hot plate

NAME

Thermofisher

BRAND

HP2310BQ

SKU

Equipment

Olympus widefield microscope, model CHK2-F-GS

NAME

Olympus

BRAND

N/A

SKU

Equipment

Cryo immuno diamond knife, 3mm

NAME

Diatome

BRAND

DCIMM3530

SKU

Equipment

Eyelash or Dalmatian hair mounted on a wooden stick

NAME

N/A

BRAND

N/A

SKU

Equipment

BRAND disposable microcentrifuge tubes, 1.5mL with safety lid

NAME

Brand

BRAND

BR780400-450EA

SKU

Equipment

SafeSeal Microcentrifuge Tube

NAME

2 mL

TYPE

Sarstedt

BRAND

72.695.500

SKU

https://www.fishersci.com/shop/products/safeseal-micro-tube-2-ml-1/NC1186931

LINK

Equipment

50 Mesh copper grids

NAME

Gilder

BRAND

GCU50

SKU

Equipment

50 Mesh palladium/copper grids

NAME

Gilder

BRAND

GCU-PD50

SKU

Equipment

150 Mesh copper grids

NAME

Gilder

BRAND

GCU150

SKU

Equipment

150 Mesh palladium/copper grids

NAME

Gilder

BRAND

GCU-PD150

SKU

Equipment

Leica EM ACE200 coater

NAME

Leica

BRAND

N/A

SKU

Equipment

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

NAME

Falcon

BRAND

FAL353047

SKU

Equipment

Perfect loop

NAME

Diatome

BRAND

70944

SKU

Equipment

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

NAME

ProSciTech

BRAND

LCH192

SKU

Equipment

3ml graduated transfer pipette

NAME

Copan

BRAND

200C

SKU

Equipment

Oven MINO/6/CLAD

NAME

Genlab

BRAND

N/A

SKU

Equipment

Sprout plus mini centrifuge NAME

Sprout BRAND

120610 SKU

Equipment

Paper towel NAME

Tork BRAND

2187951 SKU

Equipment

Foil NAME

N/A BRAND

N/A SKU

Equipment

Remanium wire loop NAME

N/A BRAND

N/A SKU

Equipment

Grid storage box, 100 grid capacity NAME

Gilder BRAND

HL065 SKU

Equipment

JEOL JEM-1400 Plus 120keV Transmission electron microscope NAME

JEOL BRAND

N/A SKU

SAFETY WARNINGS

Liquid Nitrogen is extremely cold (-196 degrees Celsius) and can cause severe burns. Personal Protective Equipment (PPE) must be worn when handling liquid nitrogen.

Uranyl acetate is mildly radioactive and extremely toxic if ingested, inhaled or in contact with abraded or cut skin. This chemical must be handled with extreme care in a fume hood using the appropriate PPE.

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

Fixation

1 All fixation steps must be performed in a fume hood wearing the appropriate personal protective equipment (PPE). The Material Safety Data Sheet (MSDS) for each chemical must be read before commencing.

Dissect out the tissue of interest on a Teflon plate or dental wax sheet using fine forceps and a scalpel blade, at room temperature. Tissue pieces should be no larger than $1mm^3$ and always submerged 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).

Mice should be perfusion fixed prior to dissection (Step 1). For brain and spinal cord samples, add 4% sucrose to all fixation solutions (Step 1 and Step 2). The addition of sucrose improves the preservation of myelin and prevents swelling of tissue during fixation.

A brain matrix can be used to cut mouse brain tissue into slices no thicker than 1mm.

2 Place the tissue in 5 ml tubes containing the fixative used in Step 1, either 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.

Different fixatives can influence the antigenicity and ultrastructure of cells and tissue. These two fixation solutions are typically tested to determine the best immunogold labelling outcome.

3 Optionally, to store samples at 4 degrees Celsius for up to one year, remove the fixative and add 1% paraformaldehyde in 0.1 M PB (pH 7.4). 4% Sucrose (cryoprotectant) should be added to the fixative for storage of brain or spinal cord tissue, or if samples are shipped by plane. Secure the 5 ml tube lid with parafilm to prevent desiccation.

Gelatin embedding and sucrose infiltration

4 Remove the fixative and wash with phosphate buffered saline (PBS), 3 x 10 mins at room temperature.

5 Quench aldehydes with 0.15% glycine in PBS for 10 mins.

6 Optionally, infuse tissue with pre-warmed 3% gelatin in 0.1M PB for 30-60 mins at 37 degrees Celsius, gently agitating. Use a heat block to maintain the temperature.

This additional gelatin infiltration step may be required to ensure adequate penetration of gelatin into larger tissue samples.

Gelatin can be made up in 0.1M PB or PBS, and different gradients and infiltration times can be used (Steps 6-8) depending on the tissue type and brand of gelatin. This medium provides support to samples so they are easier to handle and helps to create intra-and extracellular uniformity during cryosectioning.

7 Infuse the tissue with pre-warmed 6% gelatin in 0.1M PB for 30-60 mins at 37 degrees Celsius, gently agitating.

8 Infuse the tissue with pre-warmed 10 or 12% gelatin in 0.1M PB for 30-60 mins at 37 degrees Celsius, gently agitating.

9 Flat embedding can be used to minimise gelatin block shrinkage during sucrose infiltration, improving the attachment of sample blocks to the aluminium bullseye pin. This technique also facilitates mounting samples on a pin in the correct orientation for cryosectioning.

Flat embed gelatin infused tissue as follows:

9.1 Add a thin layer of pre-warmed 10 or 12% gelatin in 0.1M PB to a petri dish ensuring the surface is completely covered and solidify the gelatin at room temperature for 60 mins. The percentage of gelatin used should be the same as that used in Step 8.

9.2 Add a second layer of pre-warmed 10 or 12% gelatin in 0.1M PB on top of the first solidified gelatin layer. Place the sample in the liquid gelatin and orient it correctly using fine forceps.

9.3 Solidify the gelatin layers and sample at 4 degrees Celsius for 60 mins.

10 Steps 10-11 should be performed in a cold room.

Cut around the tissue using a razor blade or scalpel and lift out the gelatin embedded sample from the petri dish using a small spatula.

11 Place the gelatin embedded tissue on a Teflon plate or dental wax sheet and cut off excess gelatin using a razor blade or scalpel so the block is a cube or rectangular shape for orientation and mounting purposes. If a cold room is not available, cool the Teflon plate by placing it on a glass petri dish inverted on ice.

12 Optionally, fix gelatin infused tissue blocks in 0.5% paraformaldehyde in 0.1M PB for 30 mins at 4 degrees Celsius, rotating. This fixation step reduces shrinkage of blocks during sucrose infiltration/cryoprotection (Step 14) and minimises compression of frozen sections during cryosectioning. Fixed tissue blocks can be more brittle and may pop off pins during trimming if care is not taken.

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

Samples must be rinsed thoroughly to prevent aldehydes blocking antigen sites.

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

2.3M sucrose cryoprotects samples so they are vitreous when frozen. Samples shrink, dry and become brittle if left in sucrose for too long. Avoid infiltrating tissue blocks for more than 2-3 days.

Freezing

15 Samples should be mounted on a pin and frozen one at a time.

Load a clean aluminium bullseye pin onto a mounting block cooled on ice. The surface of the pin should be roughened using a pin or file prior to use to ensure the sample block adheres to the pin properly. Pins must be sonicated in acetone before use to remove oil and remnant metal filings.

16 Mount a gelatin-embedded tissue block onto the pin using fine forceps.

17 Orient the block flat and long side down using forceps, and remove the excess sucrose with a strip of filter paper so the block surface appears shiny and enough sucrose remains at the block base to cement it to the pin. Blocks larger than 1mm^3 should be mounted in the centre of the pin.

If the tissue block is too big or too much sucrose is used for mounting, a freezing imbalance between the pin and sample can lead to the formation of cracks at the base of the tissue block during freezing. Consequently, the block may break away from the pin during cryosectioning.

If a block requires reorienting, add a drop of ice cold 2.3 M sucrose in PB to the pin and repeat Step 17.

18 Freeze each tissue block by gently plunging the sample pin in liquid nitrogen. For large blocks (>1mm^3) or blocks of mostly gelatin, cool the sample pin in the cryo-ultramicrotome chamber at -100 degrees Celsius for 10 mins prior to plunge freezing in liquid nitrogen. This prevents cracks forming at the base of the tissue block due to a freezing imbalance between the pin and the sample.

Frozen samples can be stored for years.

Cryosectioning

19 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 -100 degrees Celsius using a Leica UC7/FC7 cryo-ultramicrotome (or equivalent cryo-ultramicrotome) and Diatome cryotrim 20 knife (or sharp edge of glass knife). Fixed gelatin blocks (Step 12) should be trimmed at a slightly warmer temperature (-90 degrees Celsius).

20 To check the region of interest:

20.1 Cut semi thin frozen sections with a feed of 100-500 nm, at 0.8 to 3 mm/sec and at -90 to -

100 degrees Celsius using a Diatome cryotrim 20 knife. Use a warmer sectioning temperature (-90 degrees Celsius) for fixed gelatin blocks.

20.2 Pick up semi thin frozen sections in a 1:1 mixture of 2% methyl cellulose: 2.3 M sucrose in 0.1M PB (pick-up solution) using a 3 mm stainless steel loop. For details on section retrieval, see Steps 23.1-23.3).

20.3 Transfer the sections to a glass slide by carefully pressing the loop containing the pick-up solution with sections onto the slide.

20.4 Stain the sections with methylene blue/Azure II or toluidine blue solution for 10-20 seconds on a mini hotplate before rinsing with milliQ water and drying on a mini hotplate.

Toluidine blue stains nucleic acids and proteins, enhancing structural detail of semi thin tissue sections.

20.5 Observe sections with a wide-field light microscope. If the region of interest has not been obtained, continue trimming and repeat Step 20.

21 Cut a ribbon of approximately four 50-70 nm ultrathin frozen sections at 0.8 mm/sec and -90 to -120 degrees Celsius using a Leica UC7/FC7 cryo-ultramicrotome and Diatome cryo immuno diamond knife.

The cutting temperature can be lowered for thinner sections or increased for compact or well-fixed samples. If the temperature is too high, the sample block can soften and sections may compress.

A short ribbon allows the sections to stretch in the pick-up solution once thawed, minimising the occurrence of section wrinkles.

22 Carefully detach the ribbon of ultrathin frozen sections from the diamond knife edge and drag the ribbon away from the knife edge using an eyelash or Dalmatian hair mounted on a wooden stick (one stick in each hand).

23 Pick up the ultrathin frozen sections in a 1:1 mixture of 2% methyl cellulose : 2.3 M sucrose in PB (pick-up solution) using a 3 mm stainless steel loop as follows:

23.1 Dip the stainless steel loop into a microcentrifuge tube containing the pick-up solution to create a thin film of solution over the loop. The droplet must not be too thick (large meniscus) or too thin (no meniscus).

23.2 Bring the stainless steel loop containing the pick-up solution into the cryochamber and towards the ribbon of sections. As soon as the pick-up solution starts to freeze white at the edge of the loop, move the loop forward so that the partially frozen droplet gently contacts the ribbon of sections.

Take extreme care to avoid damaging the diamond knife edge with the stainless steel loop.

23.3 Retract the stainless steel loop containing the pick-up solution with sections from the cryochamber and wait for the droplet to thaw completely.

24 Transfer the sections to a 50-150 mesh copper or palladium-coated copper grid with carbon-coated formvar film by carefully pressing the loop containing the pick-up solution with sections onto the grid.

During formvar film preparation, coated grids are secured to a sheet of parafilm wrapped around a glass slide.

25 Sample grids immersed in pick-up solution can be stored for several years in a sealed petri dish at 4 degrees Celsius until further use.

Immunogold labelling of ultrathin sections for transmission...

26 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 to the well-plate without disturbing the floating grids. Seal the 24-well plate lid with parafilm.

27 Put the plate with the floating grids in an oven set to 37 degrees Celsius for 60 minutes to remove the 2% methyl cellulose, 2.3M sucrose and 12% gelatin from the tissue sections.

If sample blocks have been fixed (Step 12), increase the temperature to 60 degrees Celsius. Immunolabelling will be compromised if the gelatin has not been adequately removed from the

tissue sections.

28 Place a sheet of parafilm on the work bench using a small amount of water underneath to keep the film flat.

29 For the following steps, use a perfect loop or fine forceps to transfer grids from one drop of solution to another, section side down at room temperature. Use approximately 100-200 µl drops for rinsing solutions and 5-10 µl drops for antibodies on the clean parafilm surface. Spin all antibodies and protein A-gold (PAG) for 30 seconds using a benchtop microcentrifuge prior to use.

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

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

31 Incubate with a primary antibody diluted in 1% BSA/PBS for 45 to 60 minutes in a dark moist chamber at room temperature. A chamber can be prepared by placing wet paper towel inside a foil-covered 15 cm glass petri dish. Place the antibody drops on a slide covered with parafilm.

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

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

33 Optionally incubate with a bridging antibody diluted in 1% BSA/PBS for 30 mins, in a dark moist chamber at room temperature.

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

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

35 Incubate with 10 nm Protein A Gold (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.

36 Rinse with PBS, 5 x 2 mins.

37 Fix with 1% glutaraldehyde in PBS for 5 mins to stabilise the immunogold labelling reaction. This step must be completed in a fume hood using the appropriate PPE.

38 Rinse with distilled water, 6 x 1 min.

If the glutaraldehyde is not rinsed out completely, aldehydes will react with uranyloxalate (Step 39) to form electron dense precipitates.

39 Stain sections in a drop of 2% uranyloxalate (pH 7.0) for 5 mins.

40 Cover a glass petri dish with parafilm using a small amount of water under the film to keep it flat. Place the petri dish on ice and add 3 large drops of 2% methyl cellulose : 4% uranyl acetate (9 ml : 1 ml) (pH 4.0) to the surface.

41 Float the grid briefly in the first two drops of methyl cellulose/uranyl acetate, then transfer to the third drop and leave for 10 mins.

42 Remove and dry the grid using the looping out method:

42.1 Clean a remanium wire loop with water and dry.

42.2 Push the loop into the methyl cellulose/uranyl acetate drop and under the grid.

42.3 Lift the grid out from the methyl cellulose/uranyl acetate drop using the loop.

42.4 Tilt the loop and grid at a 45-60 degree angle and bring the loop to a piece of filter paper. The section-side of the grid should face the filter paper.

42.5 As soon as the loop contacts the filter paper, drag it slowly along the filter paper to blot the excess liquid. A thin film of methyl cellulose/uranyl acetate should remain on the surface of the suspended grid. The slower the drag, the thinner the film. Two sheets of stacked filter paper may be required for adequate blotting. This step should be optimised and the grid film thickness checked in TEM.

42.6 Dry the grid suspended in the loop for 30 minutes at room temperature.

42.7 Remove the grid from the loop using fine forceps taking care not to tear the methyl cellulose/uranyl acetate film away from the grid. Store grids in a grid storage box.

43 Place a grid in a transmission electron microscope grid holder for high resolution imaging using a transmission electron microscope.

Example: JEOL JEM-1400 Plus TEM at 80 keV equipped with a high sensitivity bottom mount CMOS 'Flash' camera.