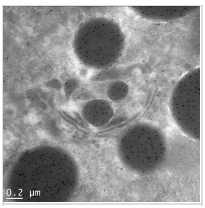

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Tokuyasu processing and immuno-electron microscopy of cells

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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 describe a protocol for the Tokuyasu technique for cryopreservation and immunolabelling of subcellular structures in cells. Cells are lightly chemically fixed and cryoprotected in sucrose before freezing in liquid nitrogen. Cells are sectioned by cryo-ultramicrotomy and retrieved cryo-sections are thawed. Thawed cryo-sections are then immunolabelled, stained and imaged using a transmission electron microscope.

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KEYWORDS

Tokuyasu, Cryosectioning, Immunolabelling, Protein-A-gold, Antibodies, Cells, Ultrastructure, Transmission electron microscope

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GUIDELINES

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

The following chemicals must be handled with extreme care in a fume hood using the appropriate PPE:

Uranyl acetate is mildly radioactive and extremely toxic if ingested, inhaled or in contact with abraded or cut skin.

Paraformaldehyde and glutaraldehyde are toxic, corrosive and potentially carcinogenic.

MATERIALS TEXT

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**

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**

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Sucrose **Merck Millipore Catalog #1.07654.1000**

Ice **Contributed by users**

Liquid nitrogen **Contributed by users**

Methylene blue **Merck Millipore Sigma Catalog #115943**

Azure II **Merck Millipore Sigma Catalog #861065**

Toluidine blue **Merck Millipore Sigma**

MilliQ Water **Contributed by users**

Methyl cellulose **Sigma Aldrich Catalog #M-6385**

Formvar powder **ProSciTech Catalog #C064**

Bovine serum albumin, heat shock fraction pH7 **Sigma Aldrich Catalog #A9647**

Mouse anti-glutamine synthetase antibody **Merck 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**

Petri dish 100mm x 20mm

Greiner Bio-One 664160

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BRAND® Petri dish, glass 150mm x 25mm

BRAND® **BR455751**

CELLTREAT Scientific Products 60mm x 15mm Petri Dish

CELLTREAT **50-828-744**

Parafilm M

Bemis **IA041**

3ml graduated transfer pipette

Copan **200C**

Dry block heater

Ratek **DBH4000D**

Corning cell scraper

Corning **CLS3011**

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Paper towel

Tork **2187951**

BRAND disposable microcentrifuge tubes, 1.5mL with safety lid

Brand **BR780400-450EA**

Sprout plus mini centrifuge

Sprout **120610**

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

ProSciTech **LCH192**

Block heater

Ratek **DBH20D**

Oven MINO/6/CLAD

Genlab **N/A**

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Single edge carbon steel blade

Electron Microscopy Sciences **71960**

SafeSeal Microcentrifuge Tube

2 mL

Sarstedt **72.695.500**

SafeSeal Microcentrifuge Tube

Fine Forceps

Forceps

Dumont **11251-10**

Black teflon plate

N/A **N/A**

Glass petri dish, 100mm

BRAND **BR455751**

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Blade scalpel ST #11

Swann Morton **21016SM**

Double edge blades

Personna **72000**

Rotary tube mixer

Ratek Instruments **RSM7DC**

Sample pin for cryo-ultramicrotomes, aluminium

Leica **75959-06**

Filter paper, grade 1, 12.5cm

Whatman **1001-125**

UC7/FC7 Cryo-ultramicrotome

Leica **EMFC7**

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Cryotrim20 diamond knife

Diatome **TT-20**

Cryo immuno diamond knife, 3mm

Diatome **DCIMM3530**

Stainless steel loop, 3mm

Contributed by user **N/A**

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

Thermo Scientific **AGL4222A**

Mini hot plate

Thermofisher **HP2310BQ**

Olympus widefield microscope, model CHK2-F-GS

Olympus **N/A**

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Eyelash or Dalmatian hair mounted on a wooden stick

N/A **N/A**

50 Mesh palladium/copper grids

Gilder **GCU-PD50**

50 Mesh copper grids

Gilder **GCU50**

150 Mesh palladium/copper grids

Gilder **GCU-PD150**

150 Mesh copper grids

Gilder **GCU150**

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

Falcon **FAL353047**

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Perfect loop

Diatome **70944**

Foil

N/A **N/A**

Remanium wire loop

N/A **N/A**

Polystyrene esky

N/A **N/A**

Leica EM ACE200 coater

Leica **N/A**

Grid storage box, 100 grid capacity

Gilder **HL065**

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JEOL JEM-1400 Plus 120keV
Transmission electron microscope
JEOL N/A

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.

Adherent cells grown in a petri dish must be submerged in solution at all times to prevent desiccation. Petri dishes should be clearly labelled on the bottom to prevent sample mix up.

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

2% Paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer:
Remove cells from the incubator and gently add a volume of double concentrate fixative, 4% paraformaldehyde, 0.4% glutaraldehyde in 0.1 M phosphate buffer (PB) equal to the volume of culture media in the petri dish. Ensure the fixative and culture media are well mixed, and incubate for 5 mins at room temperature.

4% Paraformaldehyde in 0.1 M phosphate buffer:
Remove cells from the incubator and gently add a volume of normal concentrate fixative, 4% paraformaldehyde in 0.1 M PB equal to the volume of culture media in the petri dish. Ensure

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the fixative and culture media are well mixed, and incubate for 5 mins at room temperature. Double strength fixative is not used with this fixation solution to avoid osmotic shock of cells.

2 Remove the fixative and refresh with normal strength fixative, either 2% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M PB or 4% paraformaldehyde in 0.1 M PB. Seal the petri dish with parafilm and fix cells overnight at 4 degrees Celsius.

3 Optionally, to store samples at 4 degrees Celsius for up to 1 month, remove the fixative and add 1% paraformaldehyde in 0.1 M PB. Place a sheet of parafilm directly over the petri dish, place the lid over the parafilm and seal the lid to the petri dish with more parafilm to prevent desiccation.

Gelatin embedding and sucrose infiltration

4 Remove the fixative and wash with phosphate buffered saline (PBS), 3 x 5 mins at room temperature.

5 Wash with 0.15% glycine in PBS for 10 mins.

6 Samples should be embedded in gelatin one at a time from Step 6.

Remove 0.15% glycine in PBS and add 1.5ml or 2.5 ml of 1% gelatin in 0.1 M PB (for 6 cm or 10 cm petri dishes, respectively) using a 3ml graduated transfer pipette. The gelatin should be pre-warmed to 37 degrees Celsius.

7 Gently detach adherent cells from the bottom of the petri dish using a cell scraper.

8 Transfer the cells to a 1.5 ml microcentrifuge tube using a 3 ml graduated transfer pipette, and pellet the cells immediately using a benchtop microcentrifuge.

9 Remove the supernatant using a 3 ml fine tip transfer pipette and place the closed microcentrifuge tube containing the cell pellet on a heat block set to 37 degrees Celsius.

10 Repeat Steps 6-9 for the remaining samples.

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11 Add approximately 400 µL to 1000 µL of pre-warmed (37 degrees Celsius) 12% gelatin in 0.1 M PB to the microcentrifuge tube and gently resuspend the cell pellet using a 3 ml graduated transfer pipette. Ensure no air bubbles are present and immediately place the sample on a heat block or in an oven set to 37 degrees Celsius to infuse for 20 to 30 minutes.

12 Pellet cells using a benchtop microcentrifuge and remove the supernatant leaving a volume of 12% gelatin approximately twice that of the cell pellet (ie 100 µL of 12% gelatin for a 50 µL sized cell pellet).

Cells will be stacked perpendicular to the g-forces.

13 Solidify the 12% gelatin with cells by cooling at 4 degrees Celsius for 30 minutes.

12% gelatin provides support to cells so they are easier to handle and facilitates cryosectioning.

14 Steps 14-19 should ideally be performed in a cold room to prevent condensation of water, which can affect the osmolarity of 2.3 M sucrose in PBS. If a cold room is not available, ensure the sample and sucrose are kept cool using ice.

Cut the end off the microcentrifuge tube containing approximately 30 µl of gelatin embedded cells using a razor blade and immediately submerge this in a 2 ml microcentrifuge tube containing ice cold 2.3 M sucrose in PBS.

2.3 M sucrose in PBS shrinks 12% gelatin aiding separation of the embedded cell pellet from the plastic microcentrifuge tube.

15 Cut off another piece of the microcentrifuge tube containing the remaining gelatin embedded cell pellet using a razor blade and immediately submerge this in a 2 ml microcentrifuge tube containing ice cold 2.3 M sucrose in PBS.

16 Wait a few minutes and then carefully remove the gelatin embedded cell pellet from the microcentrifuge tube tip using fine forceps. If the cell pellet does not easily separate from the plastic, return it to the 2.3 M sucrose in PBS and repeat Step 16.

17 Place a Teflon plate on top of a glass petri dish and add a drop of 2.3 M sucrose in PBS to the Teflon plate. If a cold room is not available, cool the Teflon plate by inverting the glass petri dish on ice.

18 Place the gelatin embedded cell pellet dome-side up in the drop of ice cold 2.3 M sucrose in

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PBS and cut the dome twice down the middle into three segments using a scalpel or double edged razor blade. Cut the middle segment into strips and then cut each strip into cubes no larger than $1mm^3$.

Strips can be cut into rectangles no more than 1mm wide to identify the orientation of cells.

19 Using forceps, carefully place the gelatin-embedded cell cubes or rectangles into a 2 ml microcentrifuge tube containing ice cold 2.3 M sucrose in PBS, and infuse overnight 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. Do not infiltrate cell blocks for more than 2 days.

Freezing

20 Mount each gelatin-embedded cell block on a clean aluminium bullseye pin. Orient the cell 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.

If the cell 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 cell block during freezing. Consequently, the block may break away from the pin during cryo-sectioning.

Blocks larger than $1mm^3$ should be mounted in the centre of the pin. If a block requires reorienting, add a drop of ice cold 2.3 M sucrose in PBS to the pin and repeat Step 20.

21 Freeze each sample block by gently plunging the pins in liquid nitrogen. For large blocks (>$1mm^3$) or blocks with few cells (mostly gelatin), cool the sample pin in the cryo-ultramicrotome chamber at -100 degrees Celsius for 10 mins prior to freezing in liquid nitrogen. Pins should be mounted and frozen one at a time.

Frozen samples can be stored for years.

Cryosectioning

22 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 and Diatome cryotrim 20 knife.

23 To check the region of interest:

23.1 Cut semi thin frozen sections with a feed of 100-300 nm, at 0.8 mm/sec and at -100 degrees Celsius using a Diatome cryo immuno diamond knife.

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23.2 Pick up semi thin frozen sections in a 1:1 mixture of 2% methyl cellulose: 2.3 M sucrose in 0.1M PBS (pick-up solution) using a 3 mm stainless steel loop. For details on section retrieval, see Steps 26.1-26.3).

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

23.4 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 cell sections.

23.5 Observe sections with a wide-field light microscope. If the region of interest has not been obtained, continue trimming and repeat Step 23.

24 Cut a ribbon of approximately four 60-70 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.

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

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.

25 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).

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

26.1 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).

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26.2 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 at the edge of the loop starts to freeze white, carefully press the partially frozen droplet onto the ribbon of sections.

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

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

27 Transfer the sections to 50-150 mesh copper or palladium-coated copper grids 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 on a glass slide.

28 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 electron microscopy

29 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. Seal the 24-well plate lid with parafilm.

30 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 cell sections.

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

32 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 5-10 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.

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

34 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.

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

36 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).

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

38 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.

39 Rinse with PBS, 5 x 2 mins.

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

41 Rinse with distilled water, 6 x 1 min.

If the glutaraldehyde is not rinsed out completely, it will react with uranyloxalate (Step 42) to form electron dense precipitates.

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

43 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 drops of 2% methyl cellulose : 4% uranyl acetate (9 ml : 1 ml) (pH 4.0) to the surface.

44 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.

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

45.1 Clean a remanium wire loop with water and dry.

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

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

45.4 Tilt the loop and grid at a 45-60 degree angle and bring the loop to a piece of filter paper.

45.5 As soon as the loop contacts the filter paper, drag it slowly along the filter paper to remove the excess liquid. A thin film of methyl cellulose/uranyl acetate should remain on the surface of the grid. The slower the drag, the thinner the film.

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

Remove the grid from the loop using fine forceps taking care not to tear the

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45.7 methyl cellulose/uranyl acetate film away from the grid. Store grids in a grid storage box.

46 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.

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