Tokuyasu technique for immunolabeling

Localisation and identification of molecules, proteins

**Cultured human lymphoblastoid cells:** Intracellular cholesterol was frequently detected on highly curved membranes such as tubulovesicular elements associated with the Golgi apparatus (A, arrowheads). (C) Internal vesicles of MVB appeared to be enriched in cholesterol, Bars 200 nm.

Dehydration

Critical Point Drying

Freeze-fractured/etched specimen

Freeze-dried specimen

Freeze-fracturing/Freeze-drying/Coating

RT-SEM

Freeze-substitution

Low-temperature embedding

RT-embedding

Cryo-Ultramicrotomy

RT TEM

Cryo-TEM

Cryo thin section

FROZEN SPECIMEN

Propane jet freezing

High pressure freezing

FROZEN SPECIMEN

Plunge freezing

WARM SPECIMEN

Chemical fixation

Dehydration

Critical Point Drying

Coating

Embedding

Ultramicrotomy

Staining

Immunolabeling

Replica

Freeze-dried specimen

Freeze-fractured/etched specimen

RT-SEM

RT-TEM

RT-TEM

RT-SEM

Cryo-SEM

Cryo-TEM

Preparation pathways overview
Tokuyasu technique for immunolabeling

RT specimen processing

Plunge freezing

Low temperature processing

FROZEN SPECIMEN

Cryo-Ultramicrotomy

Cryo thin section

thawing

Immunolabeling

Staining

RT-TEM

WARM SPECIMEN

Chemical fixation

Cryo-protection
Tokuyasu technique for immunolabeling

Chemical fixation → Sucrose infiltration → Plunge-freezing → Cryo-sectioning → Thawing of section → Immunolabeling → Contrast enhancement → Drying of section → TEM

Formaldehyde (low amounts of glutaraldehyde, uranylacetate)
Tokuyasu technique for immunolabeling

Chemical fixation

Sucrose infiltration

Plunge-freezing

Cryo-sectioning

Thawing of section

Immunolabeling

Contrast enhancement

Drying of section

TEM

- Formaldehyde (low amounts of glutaraldehyde, uranylacetate)
- 2.3 M Sucrose solution as cryo-protectant prevents ice-crystal formation during freezing
Tokuyasu technique for immunolabeling

- Chemical fixation
  - Formaldehyde (low amounts of glutaraldehyde, uranylacetate)
- Sucrose infiltration
  - 2.3 M Sucrose solution as cryo-protectant prevents ice-crystal formation during freezing
- **Plunge-freezing**
  - Vitrified, frozen specimen
- Cryo-sectioning
- Thawing of section
- Immunolabeling
- Contrast enhancement
- Drying of section
- TEM
Tokuyasu technique for immunolabeling

- Chemical fixation: Formaldehyde (low amounts of glutaraldehyde, uranylacetate)
- Sucrose infiltration: 2.3 M Sucrose solution as cryo-protectant prevents ice-crystal formation during freezing
- Plunge-freezing: Vitrified, frozen specimen
- Cryo-sectioning: Cryo-ultramicrotome
- Thawing of section
- Immunolabeling
- Contrast enhancement
- Drying of section
- TEM
Tokuyasu technique for immunolabeling

Cryo-ultramicrotome

Ultramicrotome

Cryo-chamber (-180°C to 60°C)

LN2 supply (Dewar)
Tokuyasu technique for immunolabeling

Cryo-ultramicrotome

Cryo-chamber side view

Cryo-chamber top view

Microtome arm with frozen specimen

Knife
Tokuyasu technique for immunolabeling

- Chemical fixation
  - Formaldehyde (low amounts of glutaraldehyde, uranylacetate)
- Sucrose infiltration
  - 2.3 M Sucrose solution as cryo-protectant prevents ice-crystal formation during freezing
- Plunge-freezing
  - Vitrified, frozen specimen
- Cryo-sectioning
  - Cryo-ultramicrotome
- Thawing of section
  - Picking up section with a droplet of frozen sucrose (2.3 M). Thawing of section at RT
- Immunolabeling
- Contrast enhancement
- Drying of section
- TEM
Tokuyasu technique for immunolabeling

1. Chemical fixation
   - Formaldehyde (low amounts of glutaraldehyde, uranylacetate)

2. Sucrose infiltration
   - 2.3 M Sucrose solution as cryo-protectant prevents ice-crystal formation during freezing

3. Plunge-freezing
   - Vitrified, frozen specimen

4. Cryo-sectioning
   - Cryo-ultramicrotome

5. Thawing of section
   - Picking up section with a droplet of frozen sucrose (2.3 M). Thawing of section at RT

6. Immunolabeling
   - Antibodies connected to gold particle (primary, secondary antibodies)

7. Contrast enhancement

8. Drying of section

9. TEM
Tokuyasu technique for immunolabeling

Main immunolabeling technique

What do we have?
Specimen section with antigens

What do we need?
Primary antibody: Highly specific IgG
Secondary antibody: IgG connected to colloidal gold or protein A-gold (gold provides contrast in EM)

Distance between antigen and gold: 20-30 nm
Unspecific binding background

Ca. 10 nm
Tokuyasu technique for immunolabeling

Grid with sections facing down

Droplet with desired solution

Parafilm

Labeling procedure: Transfer of grid containing the sections from solution to solution

Labeling buffer PBG 0.2% gelatin, 0.5% BSA (blocking buffer)

Specific antibody in labeling buffer

Washing

Etc.
Tokuyasu technique for immunolabeling

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3. Plunge-freezing
   - Vitrified, frozen specimen

4. Cryo-sectioning
   - Cryo-ultramicrotome

5. Thawing of section
   - Picking up section with a droplet of frozen sucrose (2.3 M). Thawing of section at RT

6. Immunolabeling
   - Antibodies with gold particle (primary, secondary antibodies)

7. Contrast enhancement
   - Uranyl-acetate in methyl-cellulose (staining and embedding). Embedding prevents drying artefacts.

8. Drying of section
9. TEM
Tokuyasu technique for immunolabeling

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8. Drying of section

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Tokuyasu technique for immunolabeling

RAT brown adipocyte

Tokuyasu section

Protein A-gold triple labeling
Albumin  5 nm
GLUT4   10 nm
Cath. D  15 nm

George Posthuma, University Medical Center Utrecht
Negative staining for TEM

Contrast based on heavy metals surrounding the topography of sample:
Uranyl-acetate or Phosphotungstic acid
Negative staining for TEM

Very quick method to image small particles at high resolution in the transmission electron microscope:

- Only small particles like bacteria, viruses, proteins
- No dehydration required
- Contrast based on heavy metals surrounding the topography of sample
- Very fast preparation (minutes)
Negative staining

- T4 phages on bacterium
- Bacterium with flagella
- Herpes simplex virus
- Human rotaviruses
Negative staining

Note: Be aware of artefacts