Correlative Light and Electron Microscopy using Immunolabelled Ultrathin Sections

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Specific localization of molecules within a complex biological structure

Actually the best approach in light microscopy is direct visualization with reporter molecules like GFP.

To detect GFP on EM level either photooxidation or antibodies against GFP are used.

So far for correlative light and electron microscopy only few reports exist on tags expressed in living cells using fluorophores which can photooxidize DAB: ReAsh and GFP.
ReAsH, GFP and GFP-tetracysteine

ReAsH is a membrane-permeant nonfluorescent biarsenical derivative of the red fluorophore resorufin which becomes strongly fluorescent upon binding to tetracysteine motifs in recombinant proteins expressed in living cells.

GFP: GFP recognition after bleaching (GRAB)

GFP-4C: Live observation of mannosidase II-GFP-4C and labelling with ReAsH for subsequent photoconversion for EM
ReAsH, a photooxidizable biarsenical fluorophore to image tetracysteine-tagged connexins in gap junctions

Gaietta, G. et al. (2002) Multicolor and electron microscopic imaging of connexin trafficking. Science 296, 504-507. Fig. 4.
Detection of a GFP tagged Golgi resident glycosylation enzyme, N-acetylgalactosaminy1transferase-2 (GalNAc-T2)

However, still in most studies localizing molecules on cellular level affinity labelling with antibodies and lectins is used and performed either with permeabilized samples or on sections of embedded material.

Hereby, of course, probes have to be visualized indirectly using coupled marker molecules e.g. enzymes, fluorochromes, gold particles.
Affinity localization of intracellular structures: Labelling of permeabilized samples and thick sections (Pre-embedding techniques)

Identical labelling conditions for electron microscopy as for confocal light microscopy

3D access to remaining epitopes

Drawbacks of pre-embedding techniques

Permeabilization destroys some fine structure

Loss of soluble material is an intrinsic property

Penetration problems for antibodies and markers as extraction may be uneven as well as accessibility
Affinity localization of intracellular structures: Labelling of ultrathin sections (<0.3 µm) (Post-embedding / On-section techniques)

*Thawed cryosections* of chemically fixed and sucrose-infiltrated samples according to Tokuyasu

*Ultrathin resin sections* of chemically fixed or cryofixed samples embedded in methacrylates or epoxy resins

General drawback of post-embedding techniques:

Only a *low number of antigen copies* is accessible on the section surface
Benefits and drawbacks of post-embedding labelling using thawed cryosections:

Antigens are always in an aqueous medium prior to labelling
Prefixation is the only potential denaturation step
Bad retention of soluble proteins and small molecules

using ultrathin resin sections:

Good structural preservation particularly in combination with cryoimmobilization and freeze-substitution
Resin monomers are potential skin irritants and sensitizers
Fixation, dehydration and embedding in resin may destroy the antigenicity
Visibility and sensitivity of different protein A-gold sizes

OmpA in *E. coli* wild type cells (Lowicryl K4M sections)
<table>
<thead>
<tr>
<th>Fixation</th>
<th>Dehydration</th>
<th>Resin</th>
<th>Gold/µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA/GA prefixed</td>
<td>PLT</td>
<td>K4M</td>
<td>10.60</td>
</tr>
<tr>
<td>FA/GA prefixed</td>
<td>FS methanol</td>
<td>K4M</td>
<td>15.49</td>
</tr>
<tr>
<td>Cryofixed FS</td>
<td>FA/GA methanol</td>
<td>K4M</td>
<td>19.75</td>
</tr>
<tr>
<td>Cryofixed FS</td>
<td>UA methanol</td>
<td>K4M</td>
<td>22.95</td>
</tr>
<tr>
<td>Cryofixed FS</td>
<td>Methanol</td>
<td>K4M</td>
<td>24.52</td>
</tr>
<tr>
<td>Cryofixed FS</td>
<td>Methanol</td>
<td>HM20</td>
<td>23.37</td>
</tr>
</tbody>
</table>

Relocation of OmpA during sectioning in cryofixed *E. coli* freeze-substituted in ethanol omitting any crosslinking fixatives
Relocation of OmpA during sectioning in cryofixed *E. coli* freeze-substituted in ethanol omitting any crosslinking fixatives

Cross section of a Lowicryl HM20 section labelled for OmpA
Visibility and sensitivity of different protein A-gold sizes

OmpA in *E. coli* wild type cells (Lowicryl K4M sections)
Outer membrane protein OmpA in *E. coli*

Immunofluorescence of an ultrathin Lowicryl K4M section labelled for OmpA using a 100x oil immersion objective
Post-embedding labelling offers a good combination of both, structural integrity and reliable signal for correlative light and electron microscopy.

Examples for immunolabelling of ultrathin resin sections of the very same specimen block for light and electron microscopy:

- $\alpha$-tubulin in trypanosomes
- $\beta$-catenin in intestine and heart muscle
- Recent examples for correlative microscopy:
  - Prolactin in zebrafish anterior pituitary gland
  - Chitin in *Drosophila* embryos
  - $\alpha$-tubulin, $\gamma$-COP in *Arabidopsis* pollen
  - $\alpha$-tubulin in *Drosophila* embryos and nematodes
## Strategy

<table>
<thead>
<tr>
<th>Section</th>
<th>Coverslip</th>
<th>Toluidine Blue</th>
<th>Epon</th>
<th>Bright-Field</th>
<th>Orientation and Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>500-1000 nm</td>
<td>Coverslip</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-100 nm</td>
<td>Coverslip</td>
<td>Primary Antibody</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-100 nm</td>
<td>Coverslip</td>
<td>Primary Antibody</td>
<td>Mowiol</td>
<td>Fluorescent Marker</td>
<td>Flourescence</td>
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<tr>
<td>50-100 nm</td>
<td>EM Grid</td>
<td>Primary Antibody</td>
<td>Gold Marker</td>
<td>Uranyl Acetate</td>
<td>Lead Citrate</td>
</tr>
</tbody>
</table>
Microtubules in *Trypanosoma brucei*

High-pressure frozen, freeze-substituted in osmium/acetone, embedded in Epon

Micrographs provided by Christoph Grünfelder
Tubulin in *Trypanosoma brucei*: Immunofluorescence

On-section labelling of $\alpha$-tubulin in a Lowicryl HM20-embedded sample
Tubulin in *Trypanosoma brucei*: Immunogold

On-section labelling of $\alpha$-tubulin in a Lowicryl HM20-embedded sample
The use of 500 nm thick resin sections in light microscopy

Toluidine blue stained Lowicryl K4M section of rat intestine

16x oil immersion objective

100x oil immersion objective

Toluidine blue stained Lowicryl K4M section of rat intestine
Correlative immunolabelling on methacrylate sections: β-catenin in epithelial cells of rat intestine
β-catenin in rat intestine (low mag)
β-catenin in rat intestine (high mag)
Correlative immunolabelling on methacrylate sections: β–catenin and F-actin in epithelial cells of rat intestine

- β–catenin Cy3
- propidium iodide
- F-actin FITC
- Double exposure
- β–catenin 15 nm gold
Correlative immunolabelling on methacrylate sections: β-catenin and F-actin in guinea pig heart muscle

β-catenin: Cy3 (yellow)  DNA: DAPI (blue)

Localization of $\beta$-catenin in the Z-line of heart muscle: the signal is associated with a structural correlative

Appearance of the skin of plakoglobin null-mutant mice

Appearence of desmosomes in the skin of plakoglobin null-mutant mice

Wild type (plakoglobin +/+)

Null-mutant (plakoglobin -/-)

β-catenin in the skin of plakoglobin null-mutant mice

β-catenin in the intestine of plakoglobin null-mutant mice

Prolactin labelling in the pituitary of zebrafish

Study with 7 day old fishes which were fixed with 4% FA, dehydrated in ethanol at progressively lower temperature (PLT method) and embedded in Lowicryl K11M

Prolactin labelling in the pituitary of zebrafish wt 7 dpf

Prolactin labelling in the pituitary of zebrafish wt 7 dpf

Prolactin labelling in the pituitary of zebrafish wt 7 dpf

Prolactin labelling in the pituitary of zebrafish wt 7 dpf

Spotting the region of interest on EM level

Search a 2 £ coin on a soccer field 90 x 45 m

Cuticle differentiation during *Drosophila* embryogenesis

Study using high-pressure frozen fly embryos which were freeze-substituted in 2% OsO₄, 0.5% UA, 0.5% GA in acetone (containing 2.5% methanol) and embedded in Epon.

Chitin labelling using wheat germ agglutinin (WGA)

Wild type Chitin synthase mutant CS-1/kkv (krotzkopf verkehrt)
Labelling of $\alpha$-tubulin and $\gamma$-COP in Arabidopsis pollen
$\alpha$-tubulin in Drosophila embryos and nematodes

Specimen were cryofixed by high-pressure freezing and in most cases freeze-substituted in 0.1% OsO$_4$, 0.2% UA, 0.5% GA in acetone. Samples were then washed at –35°C and rehydrated at 0°C in the presence of 0.5% and 0.25% GA.

For cryosectioning according to Tokuyasu rehydrated samples were infiltrated with sucrose/PVP and frozen in liquid nitrogen. All gold labelling was done with Nanogold or ultrasmall gold followed by silver enhancement.

α-tubulin in *Arabidopsis* pollen

Labelling of 300 nm cryosections after

Chemical fixation

HPF – FS – Rehydration

* Generative cells
α-tubulin in Arabidopsis pollen

HPF – FS – Rehydration
α-tubulin in *Arabidopsis* pollen

HPF – FS – Rehydration
γ-COP in *Arabidopsis* pollen

Labelling of 300 nm cryosections after

Chemical fixation

HPF – FS – Rehydration
γ-COP in *Arabidopsis* pollen

Labelling of 300 nm cryosections after

Chemical fixation

HPF – FS – Rehydration
$\alpha$-tubulin in the nematode *Pristionchus pacificus*

Labelling of cryosections after HPF, freeze-substitution and rehydration
α-tubulin in the nematode *Pristionchus pacificus*
α-tubulin in the nematode *Pristionchus pacificus*
α-tubulin in the nematode *Pristionchus pacificus*
Reasons for using ultrathin sections in light microscopy

Signal is restricted to the surface of a resin section: *No out-of-focus signal blurs the image*

Signal is not increased in thicker sections, but autofluorescence is

Serial sections of the same structure can be collected alternately for light and electron microscopy

High magnification objectives can be used routinely for ultrathin sections, even those stained for histology

Thin sectioning conserves rare material
Advantages of immunofluorescence over immunogold

- Rapid screening of sample areas – large field of view
- Labelling of multiple antigens is much easier with different fluorochromes than with gold particles of different sizes
- Even the smallest gold marker may influence the binding properties of an antibody more than a fluorochrome

Advantages of immunogold over immunofluorescence

- Increased resolution of EM can be exploited
- Specificity of labelling is much easier to determine due to ultrastructural information
- Gold particles allow quantification – this also serves as an additional control
Conclusions

Excellent structural preservation

Ease of orientation in stained histological tissue sections

Fast overview on labelled structures

High z-resolution in light microscopy

Direct correlation of label and antigen-containing cellular ultrastructure
Further reading:


Array Tomography Method:

- Improved resolution over confocal microscopy
- 3D volume imaging
- Consecutive staining with antibody elution
- Correlative Scanning EM using back-scattered electrons
- Automation of array tomography and puncta quantification

Array tomography

Array tomography of serial thin sections mounted on slides offers fluorescent and gold labelling to be inspected first by wide field fluorescence microscopy and then by SEM using back-scattered electrons.

Note: Automated serial block face imaging SEM (by Gatan3View/SEM or by focused ion beam milling) can so far not visualize labelled intracellular structures. (e.g. photo-oxidized DAB).
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