Assessing Ocular Morphology

in the Bioimaging Core Laboratory
Objectives

• Organization of the Bio-imaging Core Lab (BCL)

• Histological strategy for determining which aspects of the BCL might be useful to your project

  - Discuss various imaging modalities and techniques (light microscopy, transmission electron microscopy, plastic vs. paraffin vs. frozen sections, light and electron microscopic immunolabeling, confocal, deconvolution, FRET, tomography, serial block-face SEM imaging)

  - Inspire better interpretive skills, independent “critical” thinking and problem solving with microscopic material
Organization of the Bio-imaging Core Lab (BCL)

- Dr. Laura Frishman oversees all Core Labs
- Dr. Alan Burns directs and Ms. Margaret Gondo manages
- Dr. Burns assists students/faculty in histological research and design
- Ms. Gondo ensures the BCL runs smoothly, instructs students/faculty and, in some cases, assists in the implementation of their projects
• Sequence of events:

  - discuss your project with Dr. Burns and determine how the BCL can help you with your research project

  - arrange a time to meet with Dr. Burns and Ms. Gondo to discuss practical aspects of working in the BCL
A Simple Histological Strategy

**Perfusate Fixation**
- Tissue or Organ Excision
- Cell Isolation

**Fixation**
- Replaces tissue water with organic solvent in which embedding medium is soluble

**Dehydration**
- Prevents breakdown, extraction, and translocation of proteins, lipids, and carbohydrates.

**Embedment**
- Provides hard support matrix which can be sliced thin.

**Paraffin**
- Microtomy
  - Metal Knife
  - Acid-Base Histochemical
  - 8-10 um sections
  - Differential Staining
  - Transmission Light Microscopy

**Plastic**
- Ultramicrotomy
  - 0.001-0.05 um sections
  - Electron Dense Staining
  - Transmission Electron Microscopy

**Glass or Diamond Knife**

Heavy Metals
- Ua & Pb

(*)
A Simple Histological Strategy
A Simple Histological Strategy

- Perfuse Fixation
- Tissue or Organ Excision
- Cell Isolation

**Fixation**
- Coagulative (ethanol, Picric acid)
- Non-coagulative (formaldehyde, glutaraldehyde)

**Dehydration**
- Replaces tissue water with organic solvent in which embedding medium is soluble

**Embedment**
- Provides hard support matrix which can be sliced thin

- Paraffin
- Plastic

- Metal Knife
- Acid-Base Histochemical
- Microtomy 8-10 um sections
- Differential Staining
- Transmission Light Microscopy

- Ultramicrotomy 0.001-0.05 um sections
- Electron Dense Staining
- Transmission Electron Microscopy

**Prevents breakdown, extraction and translocation of proteins, lipids and carbohydrates.**
Transmission Electron Microscope
<table>
<thead>
<tr>
<th>FEATURE</th>
<th>LIGHT MICROSCOPE</th>
<th>TRANSMISSION ELECTRON MICROSCOPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>General use</td>
<td>Surface morphology and sections (1–40 μm)</td>
<td>Sections (40–150 nm) or small particles on thin membranes</td>
</tr>
<tr>
<td>Source of illumination</td>
<td>Visible light</td>
<td>High-speed electrons</td>
</tr>
<tr>
<td>Best resolution</td>
<td>ca. 200 nm</td>
<td>ca. 0.2 nm</td>
</tr>
<tr>
<td>Magnification range</td>
<td>10–1,000×</td>
<td>500–500,000×</td>
</tr>
<tr>
<td>Lens type</td>
<td>Glass</td>
<td>Electromagnetic</td>
</tr>
<tr>
<td>Image ray-formation spot</td>
<td>On eye by lenses</td>
<td>On phosphorescent plate by lenses</td>
</tr>
</tbody>
</table>
Special Techniques

- Immunolabeling (light microscope)
- Confocal microscopy
- Deconvolution microscopy
- FRET
- Immunolabeling (electron microscope)
- 3D Tomography (electron microscope)
- 3D Serial block-face sectioning (scanning electron microscope)
Immunolabeling
Immunofluorescence staining for VWF in a cremaster venule and arteriole obtained from an LPS-treated mouse. A) Red fluorescence (VWF) demonstrates markedly enhanced expression in venule. B) Dual staining with smooth muscle alpha-actin (Green) confirms smooth muscle content of arteriole. Bar = 30 um
**FIGURE 1**
Generic excitation and emission spectra for a fluorescent dye.
The Observation

A Small Sphere Imaged Through a Microscope

Point-spread
The Actual Point-Spread
Deconvolution

Point Source → Point-spread Function → Deconvolved
LSM 800-Rat Goat Rabbit-3D

Rat anti RFP + Donkey anti Rat-Alexa647 (Mueller cells) Goat anti Chat + Donkey anti Goat-Alexa543 (Amacrine cells) Rabbit anti mCar + Donkey anti Rabbit-A488 (Cone photo receptors) DNA staining: Hoechst acquired with LSM 800. Image Samples with multiple labels and overlapping emission signals.

COPYRIGHT: Sample courtesy of B. Roska, Friedrich Miescher Institute for Biomedical Research Basel, Switzerland
Confocal or “in Focus” Fluorescence
Fluorescence from Below Focus
Fluorescence from Above Focus

PMT
Cultured endothelial cells after IL-1 treatment

Colocalization?
Resolution in the Axial (Z) Dimension

Point Source → Point-spread Function → Deconvolved or Confocal
Poor Z Resolution

- Elongated blurring - spherical aberration

- Low NA lenses yield substantially lower Z resolution than XY resolution.

<table>
<thead>
<tr>
<th>NA (oil lens)</th>
<th>Z/XY Resolution Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.40</td>
<td>1.7</td>
</tr>
<tr>
<td>1.30</td>
<td>2.4</td>
</tr>
<tr>
<td>1.20</td>
<td>3.1</td>
</tr>
<tr>
<td>1.00</td>
<td>4.6</td>
</tr>
</tbody>
</table>

- Conical extensions above and below an object occur when lens cone angle is <90 degrees

- Best lenses only have a 68 degree cone angle

- Information between 68 and 90 degrees is not measured by the lens!
Light Microscope Resolution (under ideal conditions)

• X-Y plane (0.2 microns or 200 nm)
• Z axis (0.5 microns or 500 nm)
4 micron bead
4 micron bead
HUVEC after IL-1 treatment

Colocalization?
What is FRET?

- FRET is the radiationless transfer of energy from a donor fluorophore to an acceptor molecule in close proximity.
- Energy transfer results in quenching of D emission and sensitized emission from the A.
- Efficiency of the energy transfer decreases as the inverse of the sixth power of the distance separating the D and A.
Different uses of FPs for FRET microscopy

A. Intramolecular FRET: Ligand-induced conformational change in linker peptide

B. Intermolecular FRET: requires the fluorophores to be within 2 to 7 nm.
FRET imaging can help define spatial relationships

Limitations:

- The absence of FRET does not mean proteins don’t interact.

- FRET signals provide an estimate of the average distance separating the fluorophores.

- FRET does not prove a direct interaction between two proteins.

FRET imaging Limit < 7 nm

© RNDay FRETWRKSP '03
Figure 1. Immunolocalization of IL-8 in HUVEC: Golgi apparatus and Weibel-Palade bodies. HUVEC were stimulated with 1,000 U/ml IL-1β for 4 h (a–d) or overnight (e–f). Cells were fixed and permeabilized (a–f) or fixed only (g–l), and colocalization experiments were performed as described in Materials and Methods. Images (a–f) were recorded with the confocal laser scanning microscope (scale bar, 10 µm). Localization of IL-8 (a) and the 58-kDa Golgi protein (b) is shown in two separate cells and of IL-8 (d) and vWF (e) in one individual cell. Images a and b were merged to yield c, and the merged image of d and e is shown in f; yellow areas of colocalization. Pictures g–l were photographed from an Olympus IX 70 fluorescence microscope (scale bar, 20 µm): IL-8 (g and h), vWF (i and j), and DAPI (i and j).

Figure 2. Immunoelectron microscopic colocalization of IL-8 and vWF in Weibel-Palade bodies. HUVEC were stimulated with 1,000 U/ml IL-1β overnight and processed for electron microscopy as indicated in Materials and Methods. Large granules (18-nm gold) represent vWF; small granules (6-nm gold) correspond to IL-8. Short arrows, IL-8 in the Golgi apparatus; long arrows, Weibel-Palade bodies that contain both IL-8 and vWF. Top left inset. The absence of granules from Weibel-Palade bodies stained with the irrelevant antibodies rabbit anti-collagen and mouse anti-E-selectin. Bottom right inset, Weibel-Palade bodies in unstimulated HUVEC contained only vWF, but no IL-8. Scale bar, 100 nm.
3D Electron Tomography
Tomography
Tomography

• Obtain 3D info from single section

• Microtome section (400 nm) can be optically sliced at 2 nm intervals

• Ability to rotate, slice and segment the section in XYZ
3D Serial Block-face Sectioning

- Start
- Block moves 20nm up
- Knife shaves the surface
- Block-face is scanned
SEM vs TEM
SEM Resolution is near TEM resolution

SEM

TEM

Image by Anna

TEM image courtesy of Kent McDonald UCBerkeley

0.5 μm
Corneal Keratocyte Network

A

B

C

D

1 2 3 4 5
Fig 1. Diagram of the mouse cornea showing the three anatomical zones (Limbal, Paralimbal and Central) evaluated for keratocyte-keratocyte connections. Right panel illustrates serial block-face sectioning.

Fig 2. Ultrastructural 3-D renderings of the limbus (A) and the limbal segmented keratocyte network (B). Individual keratocytes exhibit a dendritic appearance and numerous X,Y, and Z keratocyte-keratocyte connections are present.

Fig 3. Ultrastructural 3-D rendering of the paralimbus (A) and the paralimbal segmented keratocyte network (B) immediately adjacent to the limbus. Dendritic keratocyte morphology is still evident as are numerous keratocytes-keratocyte X,Y and Z connections.

Fig 4. Ultrastructural 3-D rendering of the paralimbus (A) and the paralimbal segmented keratocyte network (B) positioned 200 um central from the limbus. Keratocytes appear less dendritic and while XY connections are evident there is a reduction in Z connections.

Fig 5. Ultrastructural 3-D rendering of the central cornea (A) and the central corneal segmented keratocyte network (B) 1500 um central from the limbus. Keratocytes appear very flat with fewer dendritic processes. XY contacts between keratocytes are extensive, essentially forming a continuous sheet of cells. Z connections between adjacent keratocytes layers are rare.

Fig 6. Keratocyte Z connection density is greatest at the limbus and decreases toward the center.
Corneal Epithelial Nerves
What will you image?

Time for the TOUR!