Optical and Biological Considerations for Confocal Microscopy

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Epifluorescence Microscope

Arc light source

Excitation filter

Dichroic mirror

Objective

Specimen

Barrier filter
Diffraction and Out-Of-Focus light

$d = \text{Airy disk (1 Airy unit)}$

$\phi_{\text{Airy}} = \frac{1.22\lambda}{\eta \sin \alpha}$

Out-of-focus information
Marvin Minsky’s 1957 Confocal Microscope

by the specimen and passing the second pinhole, is captured by a

Source pinhole  Specimen

Pinhole diameter = d; excludes diffraction rings
Pinhole Diameter and Confocal slice thickness
Sarastro 1989 Confocal Microscope Design

Diagram showing the components of the confocal microscope:
- Laser
- Dichroic Beam Splitter 1
- Scanner
- Objective
- Object plane
- Focus motor/“Stepper Motor”
- Lens
- Pinhole
- Detector 1
- Detector 2
Contemporary Laser scanning Confocal Microscope Design

Zeiss LSM Meta; Leica SP2, Olympus FV1000
Fluorescence Emission Properties

**DAPI**

**SYTO 11**
Discriminating Fluorescent Probes

Molecular Probes’ Alexa Dyes
Fundamental Limits to Confocal Microscopy

Registration of multiple probes

Fluorescence limitations

Sample Prep
Compensating for Varying Section Thickness at Multiple Wavelengths
Adjusting the Optical Slice Thickness

Adjust pinholes to normalize optical slice thickness

Barley Aleurone protoplast
Pinhole Adjustment yields:

- Sections in-register
- Normalized confocal slice thickness

*C. elegans*  

*Z. mays*
The problem of simultaneous UV/Vis excitation

Collimator adjustment for separate laser fibers
Separate UV & Visible light paths can image different focal planes

Transcriptional foci of 2 linked genes (488/543nm)

Nuclei (DAPI/UV)

Transcriptional foci in *Drosophila* imaged using UV & Vis excitation
UV/Vis Ex misalignment
UV-excited and Vis-excited probes imaged to the same plane

Bacteria
DAPI and Autofluorescence
A Few Other Potential Problems
Standard coverslip designations

#1 = 130–170µm
#1.5 = 160–190µm
#2 = 170–250µm
Objectives are most efficient when the correct coverslip is used.

Mismatched coverslip thickness results in decreased energy, reduced depth resolution, and axial shift in focus.
Autofluorescence

Insect leg

Z. mays
Autofluorescence

Lung, H&E

Ex 488
Em 505–550

Ex 543
Em LP560

Aldehyde-induced

??
Paraffin-embedded
Ex 488, 543
Em LP560
Photobleaching

Single scan

Multiple scans (L)
Antifade Solutions

- Commercial: “ProLong”, “SlowFade”
- DABCO 5–15mg/ml
- p-phenylenediamine 1mg/ml
- n-propyl gallate 10–50mg/ml
Traditional Fluorescent Probes

- BCECF
- DAPI
- BCECF-AM
- FDA
- PI
- Rhodamine 123
- Chlorophyll
Contemporary Fluorescent Probes: Alexa Dyes

### Table 1. Spectral characteristics of the Alexa Fluor dyes.

<table>
<thead>
<tr>
<th>Color</th>
<th>Alexa Fluor Dye</th>
<th>Abs *</th>
<th>Em *</th>
<th>Extinction Coefficient †</th>
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<tr>
<td>1</td>
<td>Alexa Fluor 350</td>
<td>346</td>
<td>442</td>
<td>19,000</td>
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<tr>
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<td>401</td>
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<td>433</td>
<td>541</td>
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<td>4</td>
<td>Alexa Fluor 488</td>
<td>495</td>
<td>519</td>
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<td>Alexa Fluor 532</td>
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<td>553</td>
<td>81,000</td>
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<td>Alexa Fluor 594</td>
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<td>647‡</td>
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<tr>
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<td>Alexa Fluor 750</td>
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<td>775‡</td>
<td>240,000</td>
</tr>
</tbody>
</table>

![Alexa 488 structure](image)
Environmental Sensitivity: pH

![Graph showing fluorescence emission against pH for different dyes: Alexa Fluor 488, Oregon Green 488, and fluorescein.](image)

![Image showing green fluorescence under microscope.](image)
Sample Preparation

- Mountant contains glycerol and antifade solution
- Seal coverslip edges well
- Remove “old” immersion oil before re-viewing
- Refreezing is not recommended
Old Oil Absorbs Water

Immersion oil $= 1.479–1.52$

$n = 1.443$

$n = 1.438$

Slide stored at $-20^\circ C \geq 1$ month
Sampling Techniques

Maize embryo sac section series
Nyquist Sampling

$\text{128x128, 4x Average}$

$\text{512x512, 4x Average}$

$Rate \geq 2f(\text{highest})$
Pixel Averaging

Averaging pixel intensity can increase signal-to-noise
The Color Lookup Table

“Let me just take a look at it.”
Imaging System Assigns Colors Based on Assumptions