Principles & Practice of Light Microscopy 5

Special Techniques
(TIRF, FRET, FRAP, FLIP, FLIM, FCS, molecular sensors...)

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For Advance Light Microscopy course
Total Internal Reflection Fluorescence (TIRF)
Problem: background light from other planes

One approach: confocal

Even more powerful solution: TIRF

*IF* the object of interest is at the surface
Total Internal Reflection

Snell’s Law: \( n_1 \sin(\theta_1) = n_2 \sin(\theta_2) \)

Aqueous sample
\( n_2 \approx 1.33–1.38 \)

Cover Glass
\( n_1 = 1.518 \)
Total Internal Reflection

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Glass
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\( \sin(\theta_{\text{crit}}) = \frac{n_2}{n_1} \)
Total Internal Reflection

Aqueous sample

\[ n_2 \approx 1.33 - 1.38 \]

Cover
Glass

\[ n_1 = 1.518 \]

Snell’s Law:

\[ n_1 \sin(\theta_1) = n_2 \sin(\theta_2) \]

\[ \theta_1 > \theta_{\text{crit}} \]

\[ \sin(\theta_{\text{crit}}) = \frac{n_2}{n_1} \]
Total Internal Reflection

Decay length vs. angle

\[ k_z = \sqrt{k_z^2 - k_x^2} = \]

\[ = \sqrt{\left(\frac{n_2}{\lambda}\right)^2 - \left(\frac{n_1 \sin(\theta_1)}{\lambda}\right)^2} \]

\[ = \frac{1}{\lambda} \sqrt{n_2^2 - n_1^2 \sin^2(\theta_1)} \]

If \( n_1 \sin(\theta_1) > n_2 \) \( \theta_1 > \theta_{crit} \)
then \( k_z \) is imaginary: \( k_z = ib \)

\[ E \propto e^{2\pi ik_z z} = e^{-2\pi bz} \]

\[ I \propto |E|^2 \propto e^{-4\pi bz} = e^{-z/z_{TIRF}} \]

\[ z_{TIRF} = 1/4\pi b = \frac{\lambda}{4\pi} \sqrt{n_2^2 - n_1^2 \sin^2(\theta_1)} \]
Total Internal Reflection

Decay length vs. angle

\[ I \propto e^{-z/z_{TIRF}} = e^{-z \frac{4\pi}{\lambda} \sqrt{n_2^2 - n_1^2 \sin^2(\theta_1)}} \]

Typical TIRF depth
\[ \approx 75-150 \text{ nm} \]

Beam NA:
\[ n_{\text{beam}}: 1.38, 1.42, 1.46 \]

Beam “NA” = \( n_1 \sin(\theta_1) \)
Two forms of TIRF microscopy

- **Prism coupled**
  - No excitation light in emission path
    ⇒ Very low background (if quartz slide)
  - Needs separate, external beam path
    ⇒ Harder to align

- **Through the objective**
  - Easy to align
  - Excitation light in emission path
    ⇒ Vulnerable to autofluorescence in the optics
  - Requires very high NA
Total Internal Reflection Fluorescence (TIRF) or Evanescent-Wave Fluorescence Microscopy

A cell (specimen): low refraction index

Reflected laser beam

Glass coverslip: high refraction index

Evanescent field 200-300 nm.

Laser beams (various angles)
Total Internal Reflection Fluorescence (TIRF) or Evanescent-Wave Fluorescence Microscopy

A cell filled with fluorescently labeled vesicles

Glass coverslip

Evanescent field
200-400 nm.

fluorescence
Identification of individual vesicles
TIRF Objectives

Typical NA 1.45–1.49

Extreme example: Olympus NA 1.65
Requires special high-index cover glass
and (volatile, toxic) immersion fluid
TIRF examples

Focal adhesions
Epifluorescence  TIRF

Single vesicle release
1 s intervals

Microtubule growth
movie
Fluorescence Resonance Energy Transfer (FRET)
FRET
Fluorescence Resonance Energy Transfer
or Förster Resonance Energy Transfer

Need: sense *interactions*
Idea: sense *distance*

Excitation energy is transferred from donor to acceptor
Efficiency depends on distance (and on angles, motion...)

[Diagram of FRET process]
FRET efficiency vs. distance

Characteristic distance $R_{\text{FRET}}$

FRET efficiency $\propto \frac{1}{1 + (R/R_{\text{FRET}})^6}$

Example: FITC $\rightarrow$ TRITC

$R_{\text{FRET}} \approx 5.5$ nm

Distance scale $\sim$ 50 times smaller than microscope resolution
FRET efficiency vs. angles

$$R_{FRET} \propto (\cos \theta_T - 3\cos \theta_D \cos \theta_A)^{1/3}$$

Angle effects less important if the fluorophores tumble freely faster than the fluorescent decay time
FRET Pairs
Förester Spectral overlap integral

\[ E_{\text{FRET}} \propto \int E_{\text{donor}}(\lambda) E_{\text{exc}}(\lambda) d\lambda \]

Protein FRET pairs:
- CFP / YFP
- Cerulean / Citrine ?
- CyPet / Ypet ?
- EGFP / MKO / cherry ?
FRET detection

Donor (CFP)  Acceptor (YFP)

Unwanted crosstalk between bands
FRET
How can one detect it?

Want to know FRET efficiency separately from concentration

• Spectrally
  • Measure intensity of donor-donor, acceptor-acceptor & donor-acceptor (FRET)

• Donor lifetime
  • Lifetime is shortened by FRET

• Acceptor photobleaching
  • Donor intensity rebounds when acceptor is removed

• Donor photobleaching
  • Quenching by FRET slows photobleaching

• Need to calibrate for background, crosstalk etc
• Environmental effects can change spectra & lifetimes
  Complicates calibration
• **Chameleons**
  Conformation change alters FRET

• **Camgaroos**
  Perturbation of a (circularly permuted) FP by conformation change of a fusion partner
Fluorescence Recovery After Photobleaching (FRAP)
FRAP
Fluorescence Recovery after Photo-bleaching

Need: to probe transport
Idea: bleach in one area, watch recovery by transport from other areas
FRAP example

GFP-Histone H1
Wildtype H1 is immobile
Partially deleted mutants exchange much faster
Fluorescence Loss In Photobleaching (FLIP)
FLIP
Fluorescence Loss in Photo-bleaching

Need: probe connectivity
Idea: bleach in one compartment, watch loss in connected compartments by exchange

Bleach one area repeatedly. Entire ER dims. ⇒ ER is contiguous
Photo Activation (PA)
Photo-Activation
(Better?) FRAP/FLIP alternative

Some fluorophores can be activated by light

• Photo-uncagable dyes
• GFP-family proteins

Look for weak light against dark background
Instead of slight dimming of bright background

Activate a small area
Watch fluorescence spread
Photo-Activatable Proteins

Off-On
- PA-GFP, PS-CFP

Color change
- Kaede, KikGR, Eos,
- Dendra (activatable by blue)

Reversibly Switchable
- asCP, KFP (tetrameric)
- Dronpa

Dendra2 demo
- Activate before
- green
- red
- after
Fluorescence Correlation Spectroscopy (FCS)

Speckle Analysis
FCS
Fluorescence Correlation Spectroscopy

Small volume → only a few molecules → random fluctuations
- Study the noise
- Conclude about random processes at different time scales

Small excitation volume
Random molecular processes:
- Diffuse in and out
- Adopt different states
- Bind or react
- Photobleach
- ...

Study the auto-(or cross-) correlation
Image Correlation Spectroscopy
Image FCS

Sense the random fluctuations of fluorescence within an image or image sequence

Much slower than point FCS, but get whole area
Can see where you are & deal with motion
Can do spatio-temporal analysis

Image series
Measure time and space variations in intensity
Image Correlation Spectroscopy

Stationary fraction
Flowing fraction
Diffusing fraction

Areas analyzed
Flow vector
○ = 10 min diffusion length

Spatio-temporal autocorrelation

0 s  5 s  10 s  15 s
Fluorescece Speckle Microscopy FSM (Clair Waterman-Storer)

Analysis of:
- Translocation of stable structure
- Assembly of new molecules
Fluorescence Life Time Imaging (FLIM)
FLIM
Fluorescence Lifetime Imaging

Measure the lifetime of the excited state

- Separate fluorophores with similar spectra
- Detect environmental parameters that affect lifetime (FRET, pH, ...)

\[ \frac{1}{e} \]
\[ \tau_1 \]
\[ \tau_2 \]
FLIM
Measurement approaches

• **Frequency domain**
  - Modulated excitation
  - Lock-in detect emission phase

• **Time domain** *(pulsed exc.)*
  - Gated intensifier
    - Photon inefficient
  - Time-correlated single photon counting
    - Very efficient
    - ≤ one photon per pulse ⇒ slow
**FLIM Examples**

Hepatocyte membrane-stained with NBD, which has a hydrophobicity-dependent lifetime (TCSPC, 3 minutes for 300x300 pixels)
Optical Tweezers
Optical Tweezers

Mechanically manipulate the specimen with light

Why?

Measuring force and displacement of a single polymerase molecule

DNA recoil after stretching
Optical Tweezers
How does it work?

Photons carry momentum
Changing photon direction requires a force
More light refracted away from high intensity region
→ Force toward intensity maximum

Alternative way to look at it:
Field energy is less in higher index
System energy depends on particle position
dE/dx = Force
Holographic Optical Tweezers

- Many traps at once
- Independently movable
- Made using a computer-controlled spatial light modulator in a pupil plane
Fiber Optics