MICROSCOPIC TECHNIQUES 2

SPECIAL TECHNIQUES BASED ON FLUORESCENCE MICROSCOPY

BIOPHYSICS 2
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SPECIAL TECHNIQUES BASED ON FLUORESCENCE MICROSCOPY

Fluorescence microscopy

- FLUORESCENCE MICROSCOPY
  light microscopy + fluorescence
  - Image formation is based on visible light (400 – 800 nm) and the use of glass lenses.
  - The object is imaged on the basis of its fluorescence emission.

Advantages:
- spectral flexibility provided by the spectral variability of fluorophores
- excellent contrast
- less invasive
- special techniques (FRAP, FRET, FLIM)
- the resolution can be improved by special tricks built in a fluorescence microscope

How can we improve the resolution of the light microscope? …can we escape physical laws?

1877 Abbe's diffraction limit
Ernst Abbe, Carl Zeiss

2014 Nobel prize in Chemistry
Stefan Hell, Eric Betzig and William Moerner

"for the development of super-reolved fluorescence microscopy"

Ernst Abbe memorial, Jena

How can we improve the resolution of the light microscope? …can we escape physical laws?

TRICKS TO REDUCE THE POINT SPREAD FUNCTION (PSF)

- CONFOCAL PRINCIPLE
- EVANESCENT WAVE EXCITATION
- STIMULATED EMISSION
- SINGLE MOLECULE LOCALIZATION
- STRUCTURED ILLUMINATION
- MULTI PHOTON EXCITATION

Confocal microscopy

Principle: conjugated focal planes

Confocal microscopy

Principle: conjugated focal planes
Confocal microscopy

**WIDEFIELD MICROSCOPY**

- one plane in focus
- all the planes contribute to the image

**OBJECTIVE**

- light source
- fluorescence emission: focal plane
- fluorescence emission: in front of the focal plane
- fluorescence emission: behind the focal plane

**CONFOCAL MICROSCOPY**

- one plane is in focus
- ↓ APERTURE (pinhole): spatial filter
- conjugated focal plane
- one plane contributed to the image

- size of the aperture ~ 1 Airy unit
- Airy unit: diameter of the Airy disk

**3D imaging**

- optical slices
- ↓ many 2D image
- ↓ 3D IMAGE

**Confocal microscopy**

**3D imaging**

- OPTICAL SLICES
- ↓ MANY 2D IMAGE
- ↓ 3D IMAGE

**Evanescent wave microscopy**

**Principle: total internal reflection**

TIRFM – total internal reflection fluorescence microscopy
Evanescent wave microscopy

total internal reflection

\[
\alpha = \frac{n_1}{n_2} \sin(\theta_{\text{critical}}) < 1
\]

\[
\alpha = \frac{n_3}{n_4} \sin(\theta_{\text{critical}}) > 1
\]

\[
I(x) = I_0 \exp\left(-\frac{x^2}{2}\right)
\]

\[
d(\text{TIRFM}) = \frac{\lambda}{4 n_4 \sqrt{n_4^2 \sin^2(\theta_{\text{critical}}) - n_2^2}}
\]

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Stimulated emission microscopy

Principle: depletion by stimulated emission

STED – stimulated emission depletion microscopy

2014 Nobel Prize
Stefan Hell, Eric Betzig és William Moerner

"for the development of super-resolved fluorescence microscopy"

http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/

Stimulated emission microscopy

resolution widefield STED
lateral, nm 200 50
axial, nm 800 500

\[
d(\text{STED}) = \frac{\lambda}{2 N A \left(1 + \frac{1}{M}ight)}
\]

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Single molecule localisation microscopy

Principle: PSF fit by a gaussian function

PALM – photo-activated localization microscopy
STORM – stochastic optical reconstruction microscopy

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Single molecule localisation microscopy
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Photoconvertible fluorophores

Photoregulable fluorophores

Multi photon microscopy
Principle: multi photon excitation

Multi photon microscopy
Principle: multi photon excitation
Multi photon microscopy

ADVANTAGES
- improved 2 resolution
- improved penetration depth (higher wavelength – lower scattering)
- confocal: μm / two-photon: mm
- less invasive (higher wavelength – lower energy)
- imaging tissues in live animals: intravital microscopy
- deep-tissue imaging: imaging deeper layers in the sample

FRAP – fluorescence recovery after photobleaching

Principle: photobleaching/fading + diffusion

FRAP – fluorescence recovery after photobleaching

PHOTOBLEACHING
- irreversible photochemical destruction of the fluorophore due to the excitation
disadvantages
- anti-photobleaching medium (pl. glucose oxidase – catalase – mercaptoethanol)
- lower exposure time
- lower excitation intensity
- resistant fluorophore
advantages
- FRAP

FRAP – fluorescence recovery after photobleaching

http://en.wikipedia.org/wiki/Fluorescence_loss_in_photobleaching
http://micro.magnet.fsu.edu/primer/java/fluorescence/photobleaching/

PHOTOBLEACHING
FLUORESCENT BLEACHING: laser NONFLUORESCENT

RECOVERY: DIFFUSION FLUORESCENT + NONFLUORESCENT

FRAP – fluorescence recovery after photobleaching

http://micro.magnet.fsu.edu/primer/java/fluorescence/photobleaching/

FRAP

sec 01.6
see 01.8 CUNH WT

Figure 2. Localization of excitation by two-photon excitation. (a) Single-photon excitation of fluorescent by focused 488-nm light (0.36 N.A.). (b) Two-photon excitation using focused (0.36 N.A.) femtosecond pulses of 950-nm light.

Figure 9. Graph showing FRAP kinetics analysis.

Figure 3. Blood flow in the liver of a living mouse.
FRAP

Summary

Modern fluorescence microscopy keywords

- Principles of resolution enhancing techniques
  - Confocal microscopy
  - TIRFM
  - STED
  - Single molecule localization
- Principles of special imaging techniques
  - Multi-photon microscopy
  - FRAP

Recommended web resources

http://www.olympusmicro.com/index.html
http://www.microscopyu.com
http://zeiss-campus.magnet.fsu.edu/index.html
http://www.ibiology.org/