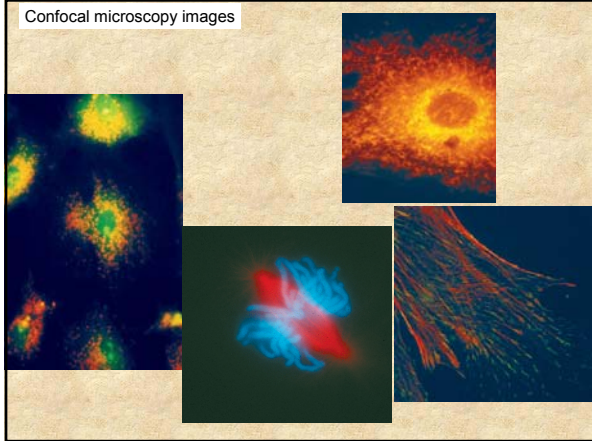


**Prof. Enrico Gratton - Lecture 6
Fluorescence Microscopy**

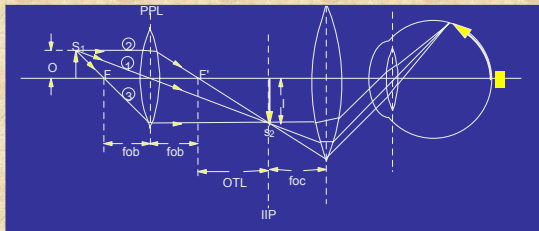
Instrumentation
Light Sources:
One-photon and Multi-photon Excitation
Applications in Cells
Lifetime Imaging

Figures acknowledgements: E.D. Salmon and K. Jacobson

Confocal microscopy images



In the compound microscope the Finite Corrected Objective Forms a Real Image At the Ocular Front Focal Plane: The Primary or Intermediate Image Plane (IIP)



Conventional Optics
Objective with finite Focal Length
(Optical Tube Length, OTL, Typically 160 mm)
 $M_{ob} = OTL/f_{ob}$

Total Magnification = $M_{ob} \times M_{oc} = OTL/f_{ob} \times 250mm/f_{oc}$

Why is the eyepiece necessary?
 E.D. Salmon
Resolution Limitations of the Human Eye

Limits to Accommodation

Unresolved Resolved

250 mm
Conventional Viewing Distance

Resolution Test

COARSE FINE

A word about infinity corrected optics and its advantages.

Infinity-Corrected Objective System

Eye Diaphragm Plane

Eye Diaphragm

Intermediate Image

Tube Lens

"Infinity Space"

Objective

Front Focal Plane of Objective

Specimen

Parallel Light Beam

Figure 3

Modern microscope component identification

Prisms Used to Re-Direct Light In Imaging Path While Mirrors Are Used in Illumination Path

Modern Microscope Component Configuration

Camera

Camera Film Plane

Focusing Viewfinder

Automatic Exposure Monitor

Figure 1

Eyepieces

Projection Lens

Binocular Prisms and Beamsplitter

Nosepiece

Objective

Stage

Condenser

Field Diaphragm

Stand

Focus Knob

Lamphouse

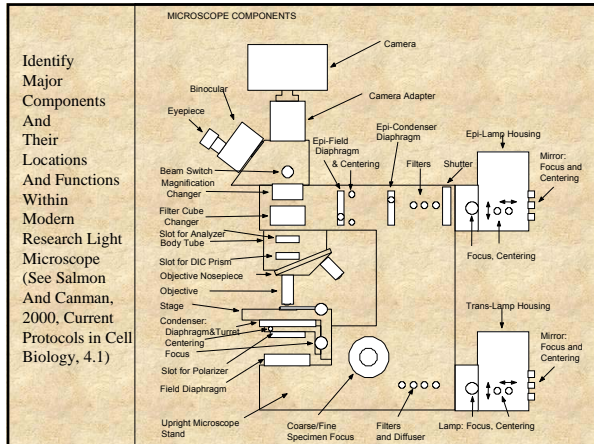
Base

Filter

Lamp

Collector Lens

E.D. Salmon



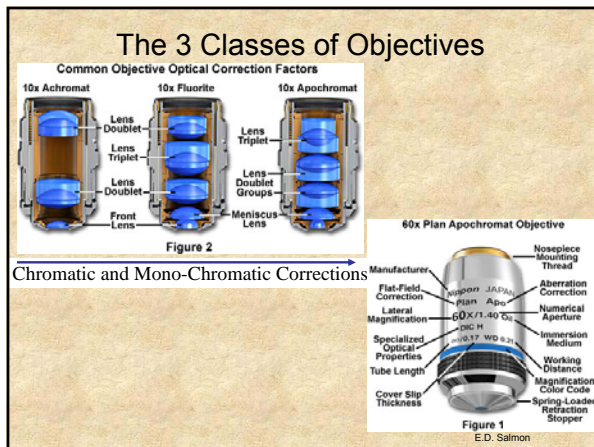
Key component: the objective

Achromats: corrected for chromatic aberration for red, blue

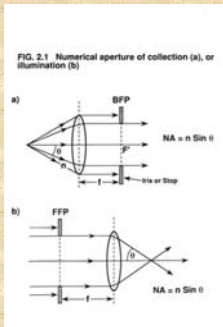
Fluorites: chromatically corrected for red, blue; spherically corrected for 2 colors

Apochromats: chromatically corrected for red, green & blue; spherically corrected for 2 colors

Plan-: further corrected to provide flat field

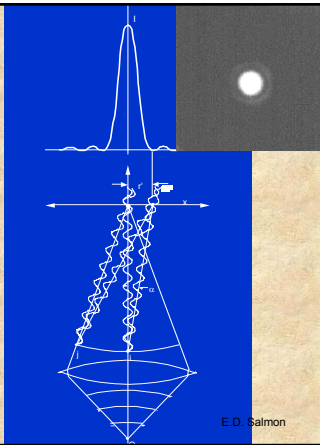


What is numerical aperture (NA)?

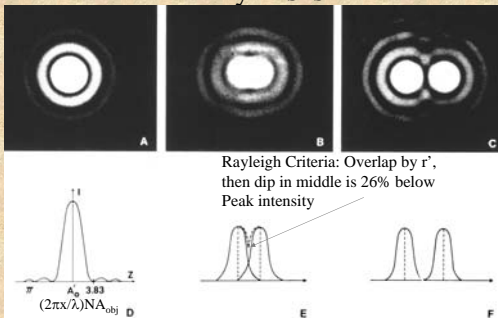


- Image Intensity: $I \sim NA_{obj}^2 / M_{tot}^2$
- Image Lateral Resolution for Corrected Objective:
 - Fluorescence: $r = 0.61\lambda / NA_{obj}$
 - Trans-Illumination: $r = \lambda / (NA_{obj} + NA_{cond})$

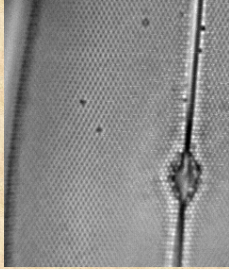
Airy Disk Formation by Finite Objective Aperture:
 The radius of the Airy Disk at the first minimum, r' , occurs because of destructive interference; the diffraction angle, α , is given by: $\sin(\alpha) = 1.22\lambda / D$, where D = diameter of objective back aperture



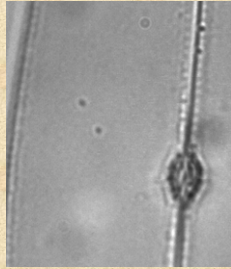
Lateral Resolution in Fluorescence Depends on Resolving Overlapping "Airy Disks"



Resolution is better at shorter wavelengths, higher objective NA or higher condenser NA



High NA and/or shorter λ



Low NA and/or longer λ

Rayleigh Criterion for the resolution of two adjacent spots:

$$P_{lim} = 0.61 \lambda_o / NA_{obj}$$

Examples: ($\lambda_o = 550 \text{ nm}$)

| | Mag | f(mm) | n | a | NA | $P_{lim} (\mu\text{m})$ | ($NA_{cond}=NA_{obj}$) |
|----------|------|-------|------|------|------|-------------------------|--------------------------|
| high dry | 10x | 16 | 1.00 | 15 | 0.25 | 1.10 | |
| | 40x | 4 | 1.00 | 40 | 0.65 | 0.42 | |
| oil | 100x | 1.6 | 1.52 | 61 | 1.33 | 0.204 | |
| | 63x | 2.5 | 1.52 | 67.5 | 1.40 | 0.196 | |

Why oil immersion lenses have greater resolution

Oil Immersion and Numerical Aperture

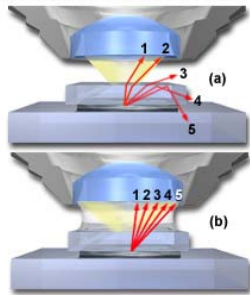


Figure 1

$$D = 0.61 \lambda \cos \alpha / n(NA)^2$$

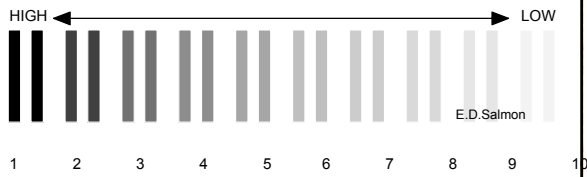
Low power, NA~0.25 D~ 8 μm

Hi, dry, NA~0.5 D~ 2 μm

Oil immersion, NA~1.3 D~0.4 μm

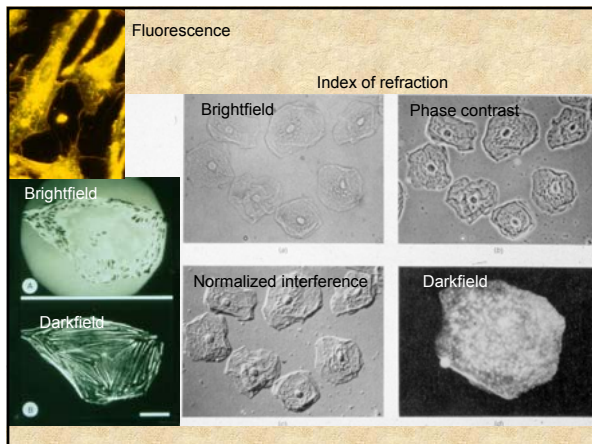
Contrast : All the resolution in the world won't do you any good, if there is no contrast to visualize the specimen.

$$\text{CONTRAST} = (I_{sp} - I_{bg})/I_{bg}$$

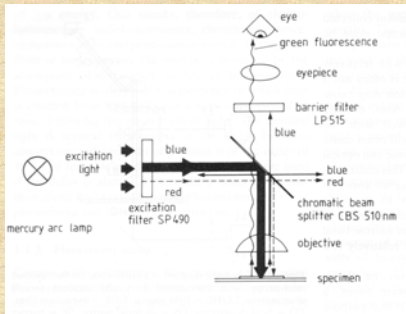


CONTRAST MODES OF LIGHT MICROSCOPY

| MODE | MECHANISM OF CONTRAST |
|----------------------------------|---|
| Brightfield | Absorption of light |
| Phase contrast | Optical path length (index, density) |
| DIC | Rate of change of optical path |
| Widefield fluorescence | Absorption of light, quantum yield of fluorophore |
| Confocal fluorescence | same as fluorescence |
| Darkfield | light scattering by edges in specimen |
| Interference reflection contrast | interference between reflections from ventral cell surface and substratum |
| Polarization | Extinction between crossed polars caused by specimen birefringence |



Basic design of the epi fluorescence microscope



Objectives

High transmittance

Fluorite lenses: $\lambda > 350$ nm [ok for FURA]

Quartz lenses: $\lambda < 350$ nm

Employ simple, non plan lenses to minimize internal elements.

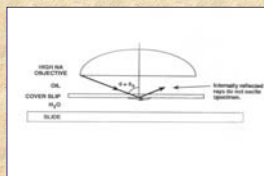
Negligible auto-fluorescence or solarization [color change upon prolonged illumination]

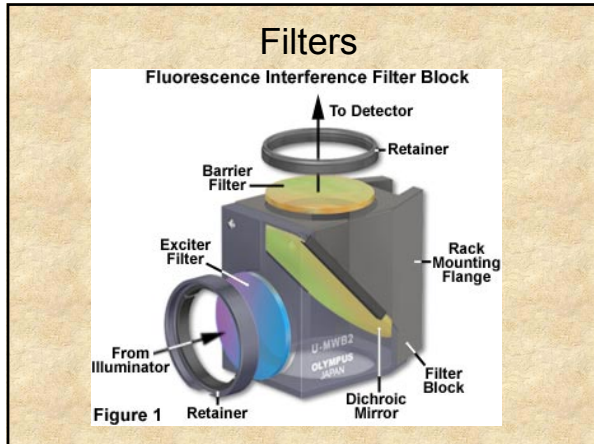
Maximizing image brightness (B)

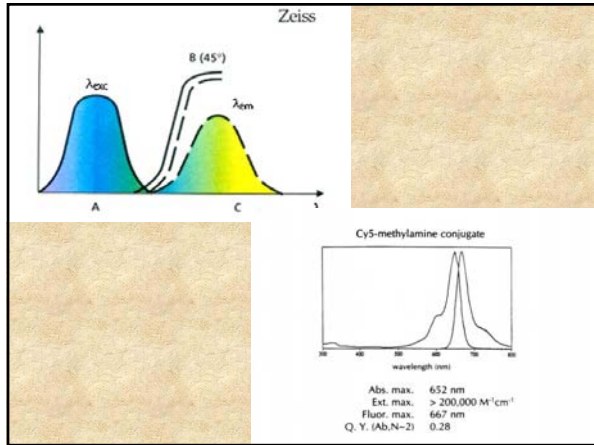
$$\left. \begin{array}{l} \text{excitation efficiency} \sim (NA)^2 \\ \text{collection efficiency} \sim (NA)^2 \end{array} \right\} \Rightarrow B \sim (NA)^4$$

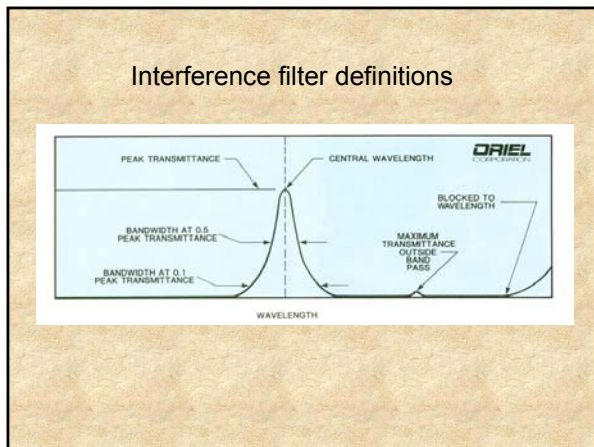
$$\text{also } B \sim \frac{1}{M^2} \Rightarrow B \sim \frac{(NA)^4}{M^2}, \text{ for } NA \leq 1.0$$

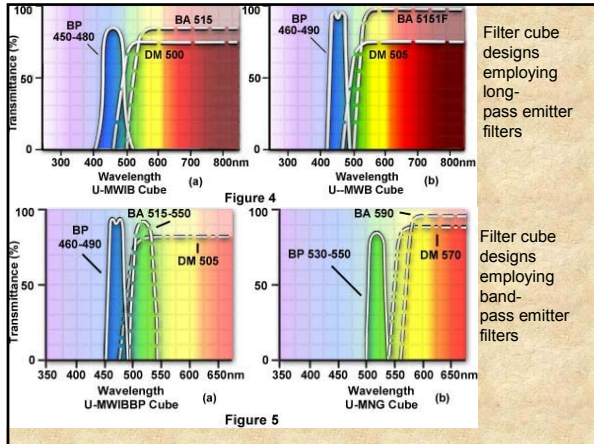
at high NA,

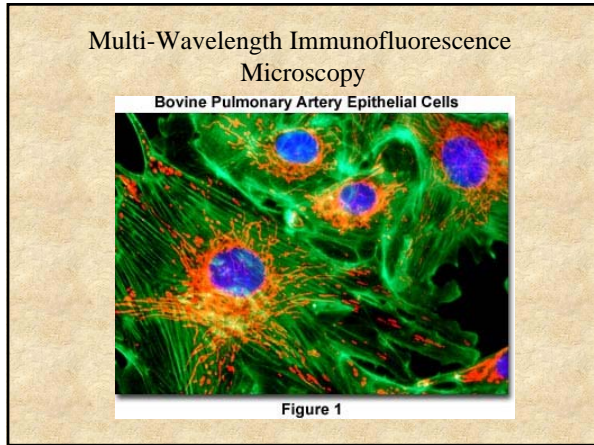


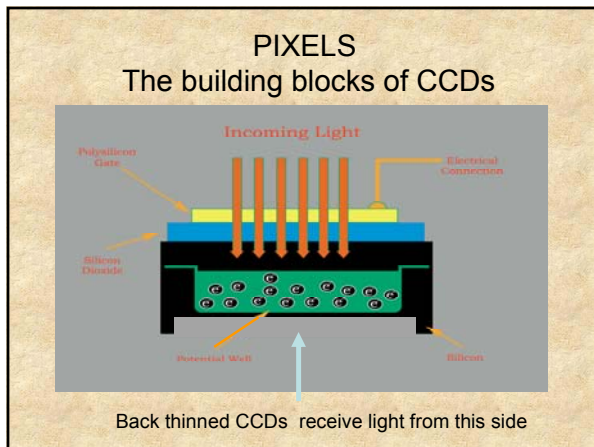












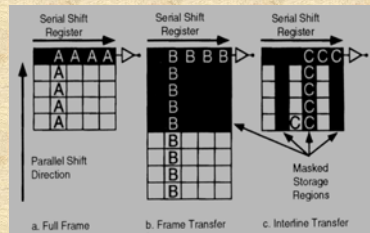
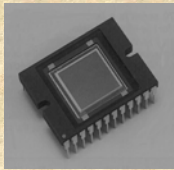
Primary Features of CCD

- **Spatial resolution of the CCD array**
 - Number of Pixels in X and Y
 - Center to Center Distance of Pixels in microns
- **Full Well Capacity**
 - Related to Physical size and electronic design
 - Determines Maximum Signal level possible
- **Quantum Efficiency/Spectral Range**
 - Determines the usefulness of the camera
 - Major influence on exposure time
- **Camera Noise**
 - The limiting feature in low light applications
 - Influenced by Readout Speed / Readout Noise
 - Influenced by Dark Current / Time
- **CCD Chip Design**
 - Influences Total Frame Rate
 - Exposure time plus Readout time
 - Total Photon Efficiency
 - Quantum Efficiency and Exposure Cycle

B. Moomaw, Hamamatsu Corp.

Types of CCD Detectors

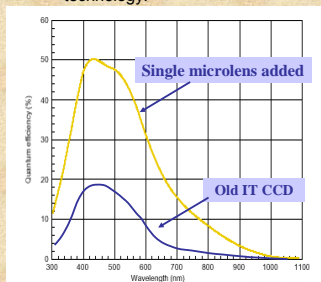
- CCD Cameras - 3 Primary Designs



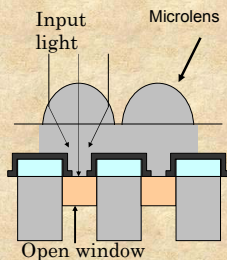
B. Moomaw, Hamamatsu Corp.

Improvements in Interline CCDs

- Effective Q.E. was greatly increased by Microlens technology.



B. Moomaw, Hamamatsu Corp.



Photobleaching

- *Photochemical lifetime*: fluorescein will undergo 30-40,000 emissions before bleaching. ($QY_{\text{bleaching}} \sim 3 \cdot 10^{-5}$)
- At low excitation intensities, photobleaching occurs but at lower rate.
- Bleaching is often photodynamic--involves light and oxygen.

Parameters for Maximizing Sensitivity

- Use High Objective NA and Lowest Magnification:
 $I_{\text{fl}} \sim I_{\text{fl}} NA_{\text{obj}}^4 / M_{\text{tot}}^2$
-Buy the newest objective: select for best efficiency
- Close Field Diaphragm down as far as possible
- Use high efficiency filters
- Use as few optical components as possible
- Match magnification to camera resolution:
 $M_{\text{Max}} = 3 \cdot \text{Pixel Size of Detector/Optical Resolution}$
E.g.: $3 \cdot 7 \mu\text{m} / [0.6 \cdot 520\text{nm} / 1.4] = 91\text{X}$
- Reduce Photobleaching
- Use High Quantum Efficiency Detector in Camera

Adapted from E.D. Salmon

Live Cell Considerations

- Minimize photobleaching and photodamage (shutters)
- Use heat reflection filters for live cell imaging
- Image quality: Maximize sensitivity and signal to noise (high transmission efficiency optics and high quantum efficiency detector)
- Phase Contrast is Convenient to Use with Epi-Fluorescence
 - Use shutters to switch between fluorescence and phase
 - Phase ring absorbs ~ 15% of emission and slightly reduces resolution by enlarging the PSF

Adapted from E.D. Salmon

Defining Our Observation Volume: One- & Two-Photon Excitation.

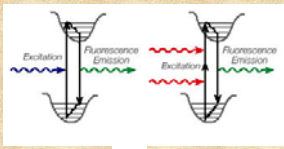

1 - Photon

Defined by the pinhole size, wavelength, magnification and numerical aperture of the objective

2 - Photon

Defined by the wavelength and numerical aperture of the objective

Approximately $1 \mu\text{m}^3$

Brid Amos
MRC, Cambridge, UK

Advantages of two-photon excitation

- 3-D sectioning effect**
- Absence of photo bleaching in out of focus regions**
- Large separation of excitation and emission**
- No Raman from the solvent**
- Deep penetration in tissues**
- Single wavelength of excitation for many dyes**
- High polarization**

Why confocal detection?

Molecules are small, why to observe a large volume?

- Enhance signal to background ratio
- Define a well-defined and reproducible volume

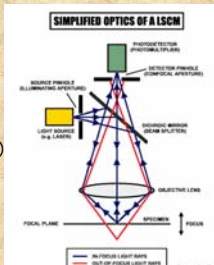
Methods to produce a confocal or small volume

(limited by the wavelength of light to about 0.1 fL)

- Confocal pinhole
- Multiphoton effects
 - 2-photon excitation (TPE)
 - Second-harmonic generation (SGH)
 - Stimulated emission
 - Four-way mixing (CARS)

(not limited by light, not applicable to cells)

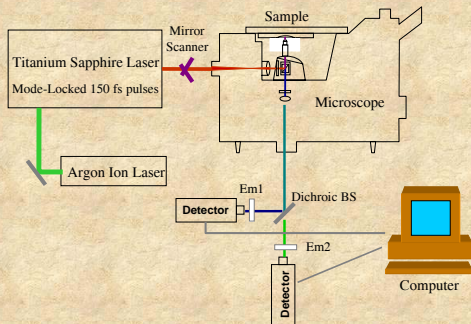
- Nanofabrication
- Local field enhancement
- Near-field effects



Lance Ladell
Ladell Optics Ltd.

How does one create an observation volume and collect the data?

Two-Photon, Scanning, FCS Microscope



Laser technology needed for two-photon excitation

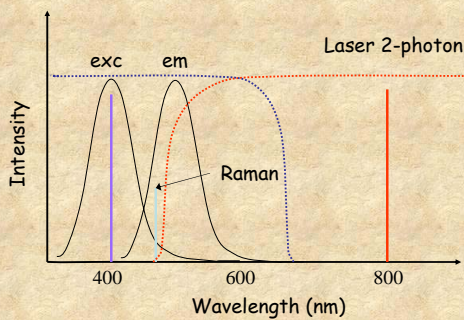
Ti:Sapphire lasers have pulse duration of about 100 fs
 Average power is about 1 W at 80 MHz repetition rate
 About 12.5 nJ per pulse (about 125 kW peak-power)
 Two-photon cross sections are typically about

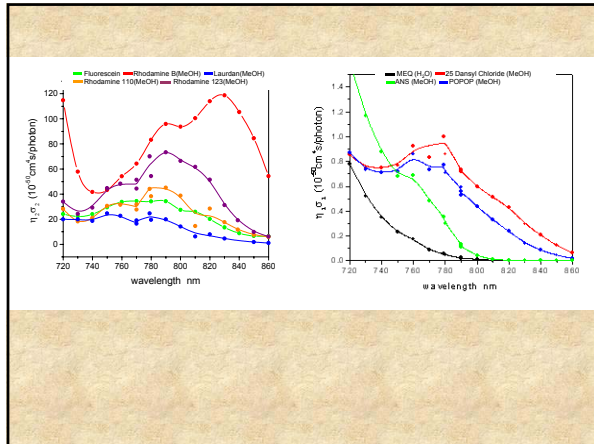
$$\delta \approx 10^{-50} \text{ cm}^4 \text{ sec photon}^{-1} \text{ molecule}^{-1}$$

Enough power to saturate absorption in a diffraction limited spot

$$n_a \approx \frac{d}{\tau} \left(\frac{p \pi A^2}{f h c \lambda} \right)^2$$

- n_a Photon pairs absorbed per laser pulse
- p Average power
- τ pulse duration
- f laser repetition frequency
- A Numerical aperture
- λ Laser wavelength
- d cross-section





General References

- Salmon, E. D. and J. C. Canman. 1998. Proper Alignment and Adjustment of the Light Microscope. Current Protocols in Cell Biology 4.1.1-4.1.26, John Wiley and Sons, N.Y.
- Murphy, D. 2001. Fundamentals of Light Microscopy and Electronic Imaging. Wiley-Liss, N.Y.
- Keller, H.E. 1995. Objective lenses for confocal microscopy. In "Handbook of biological confocal microscopy", J.B.Pawley ed. , Plenum Press, N.Y.

On line resource:

Molecular Expressions, a Microscope Primer at:

<http://www.microscopy.fsu.edu/primer/index.html>
