2017

Seeing is Believing
Symposium

Basics of Fluorescence Microscopy

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WRH Cell Imaging Facility

6 Sept 2017

westmead research hub

The Westmead Institute FOR MEDICAL RESEARCH

Light microscopy

Bright field microscopy

Fluorescence microscopy

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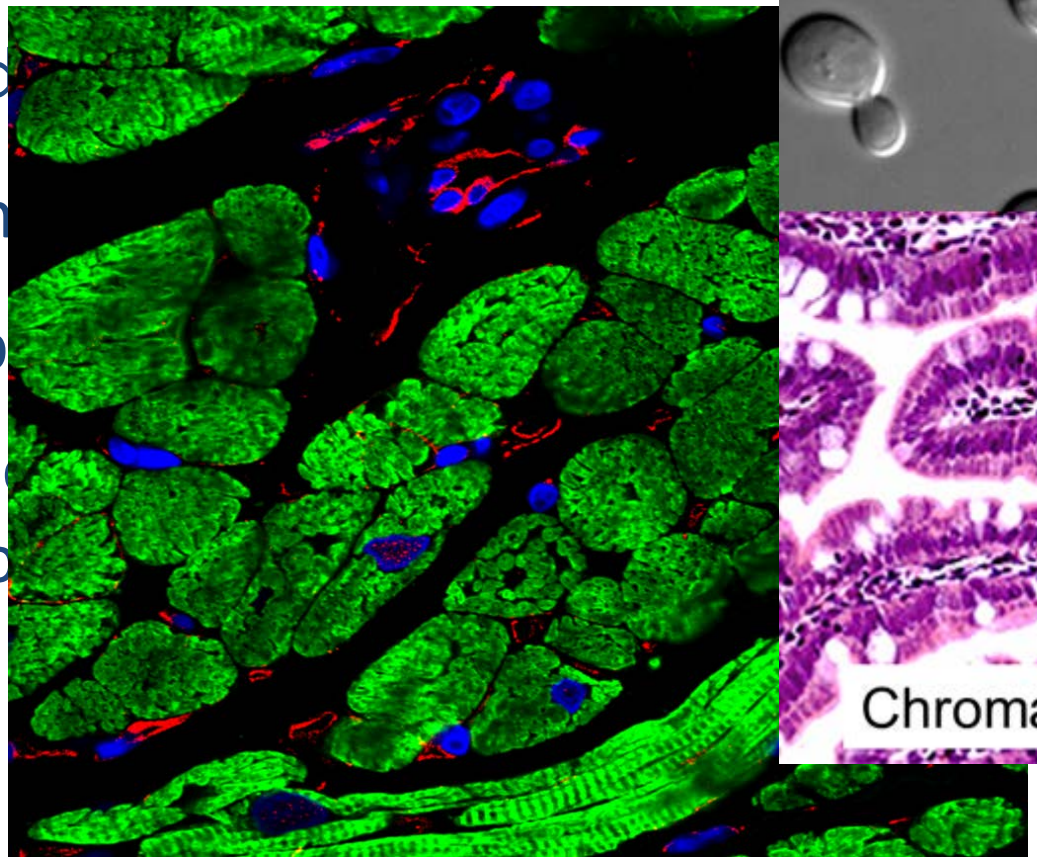
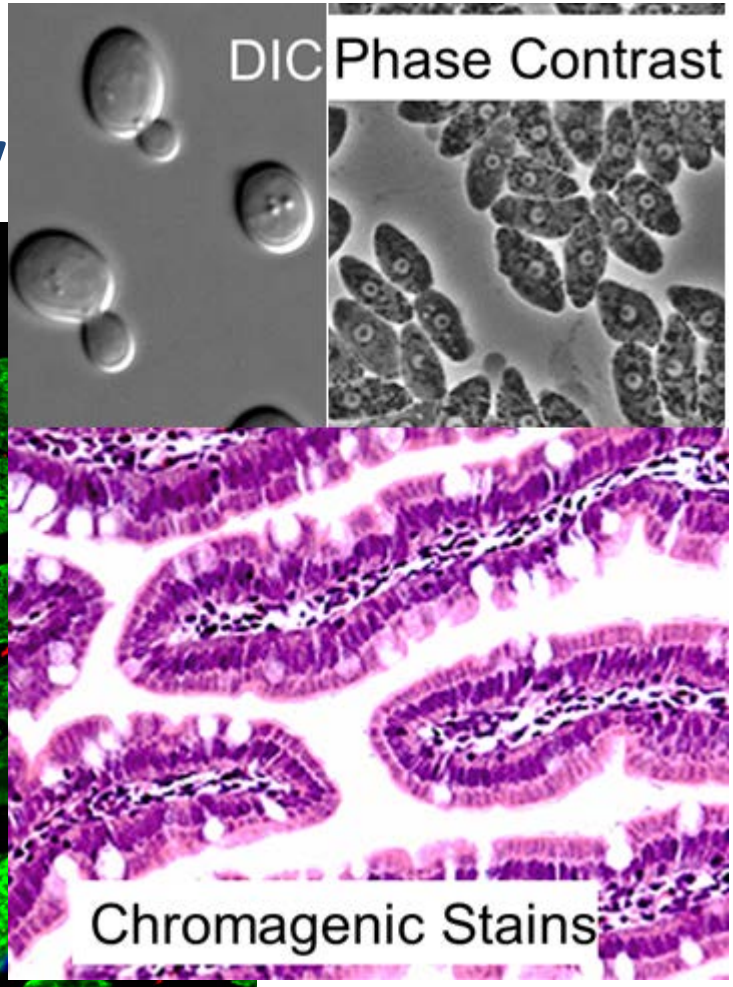


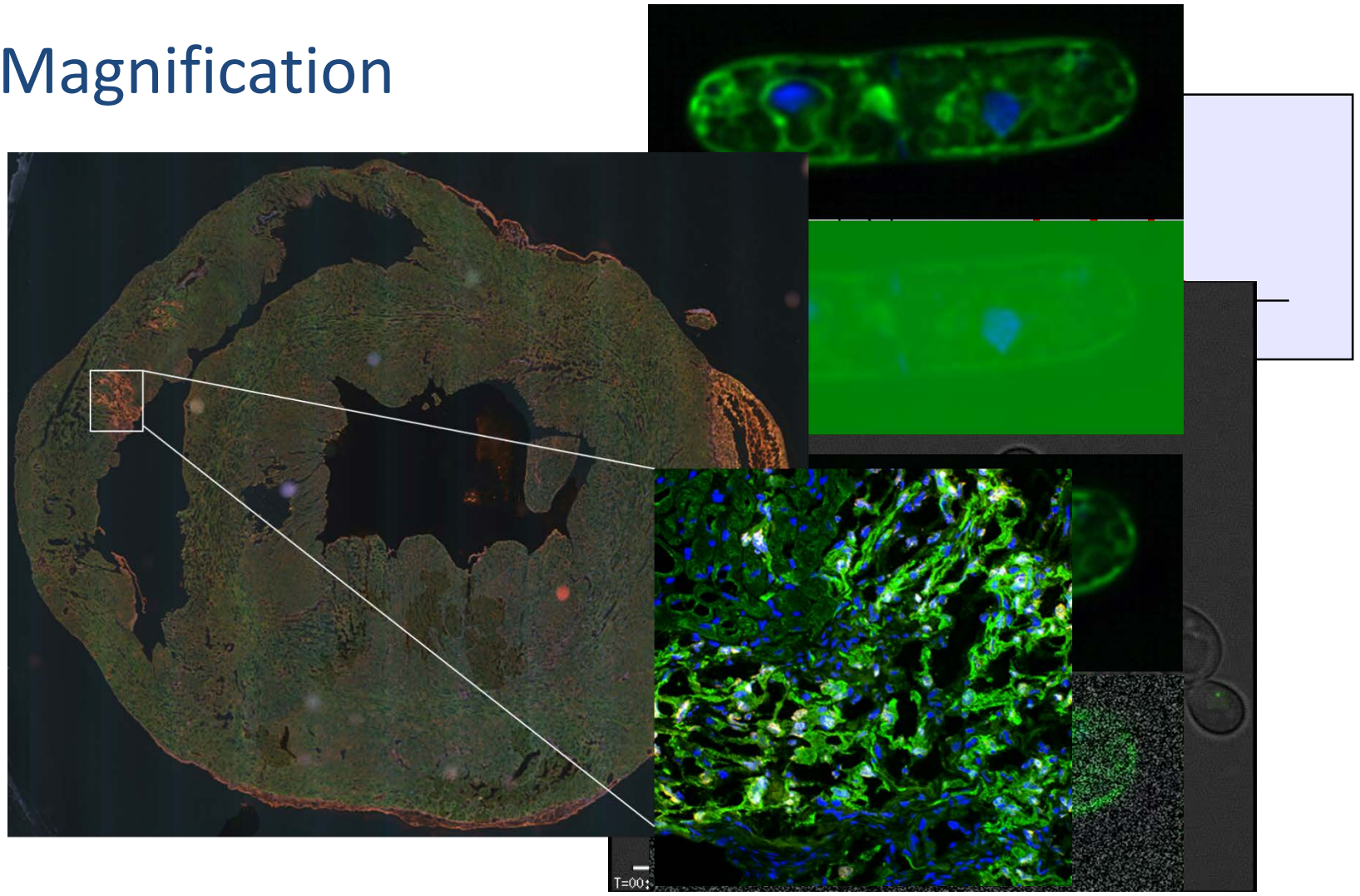


Basics of fluorescence microscopy

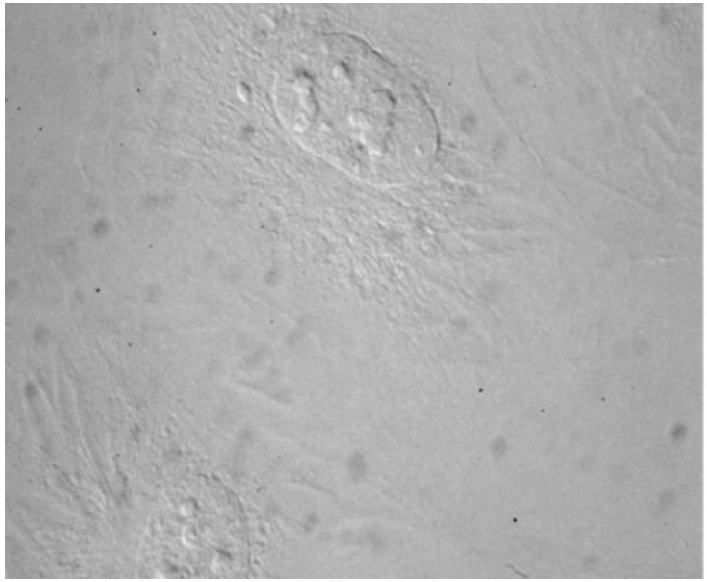
1. What is expected from a microscope?
2. Why fluorescence microscopy?
3. Theory of fluorescence
4. Fluorescence filter set
5. Understanding objectives
6. Designing IF experiments

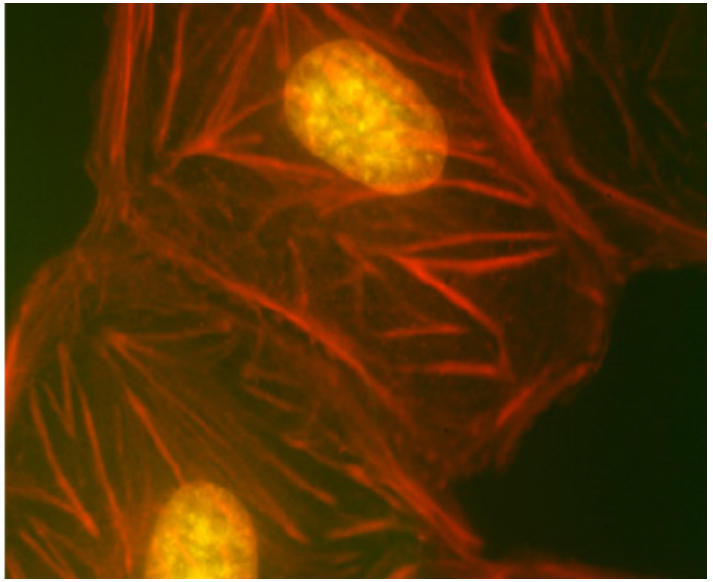
What is expected from a light microscope?

- Magnification
-
-
-



Why fluorescence microscopy?

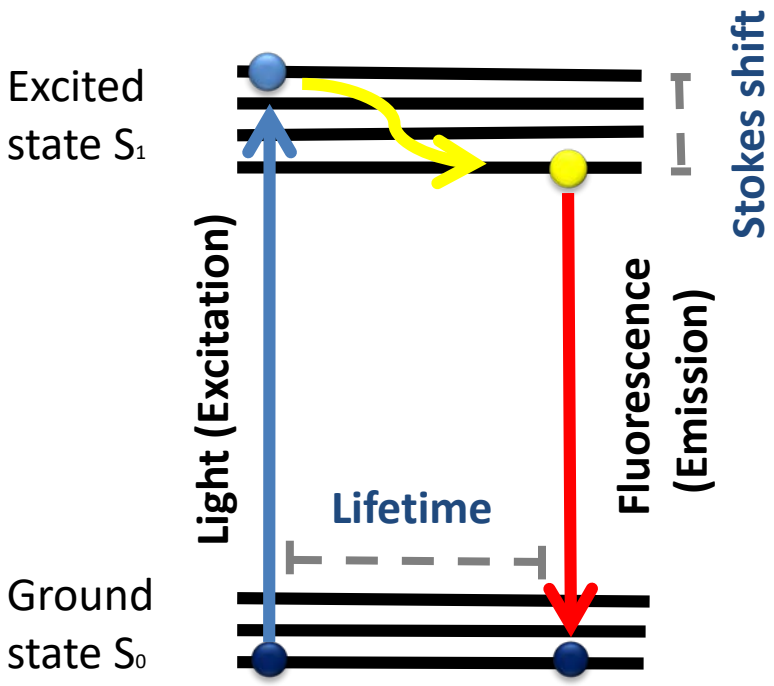


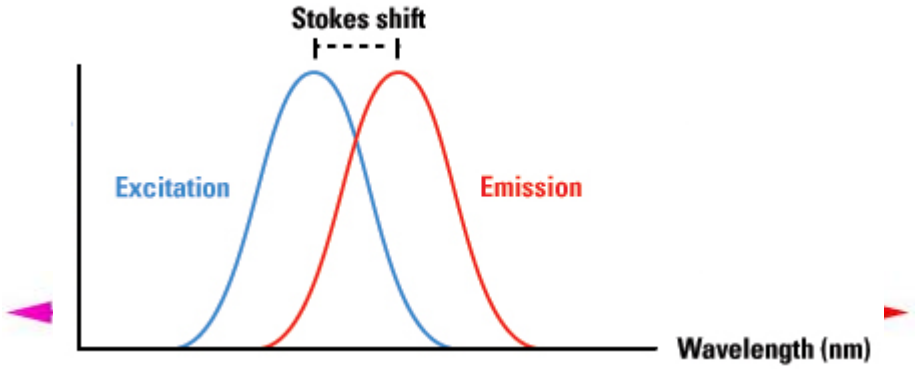


- High contrast
- High specificity
- Quantitative
- Live cell imaging
- Optical sectioning & 3D imaging
- Other advanced imaging technologies
  - FRET, FRAP, FLIM etc
  - Super res
  - Multi photon
  - Others

Theory of fluorescence

QE: quantum efficiency

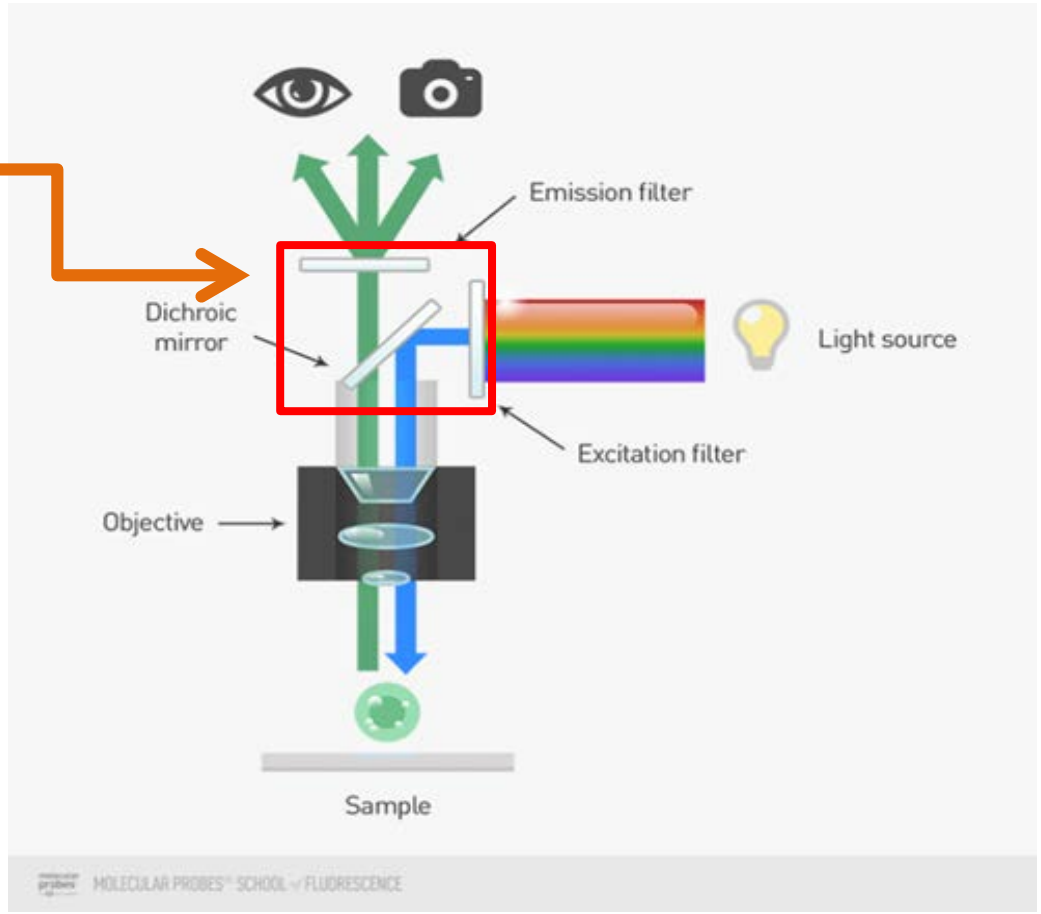




Shorter wavelength
Higher frequency
Higher energy
Weaker penetration

Longer wavelength
Lower frequency
Less energy
Better penetration

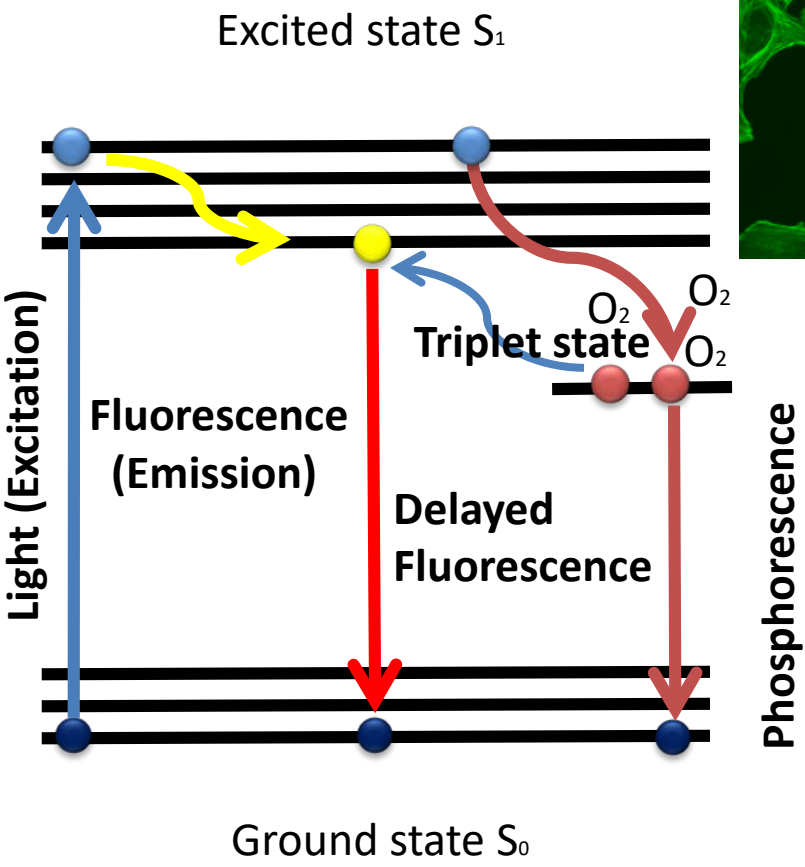
Fluorescence filter set

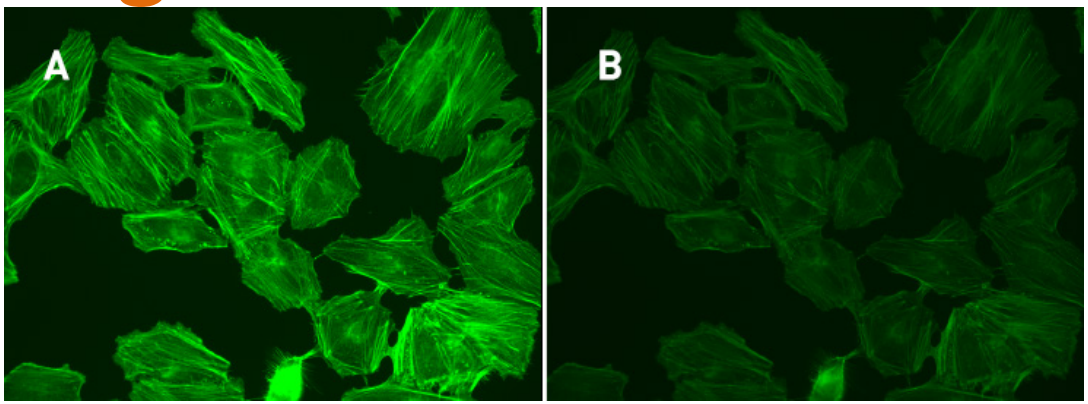


Selection of fluorescent probes

- Great Stokes shift
- High QE
- Thick/thin sample---longer/shorter wavelength
- Resolution = (0.61 x λ)/ NA shorter wavelength better resolution
- Matching filters and fluorophores---SpectraViewer

Photobleaching and solutions





Protecting against photobleaching

- **Select fade-resistant dyes**
- **Label densely**
- **Use anti-fade agents**
- **Budget the photons**:
  lower illumination,
  minimise exposure time
  only expose when observing
- **Store your slides at low temperature**

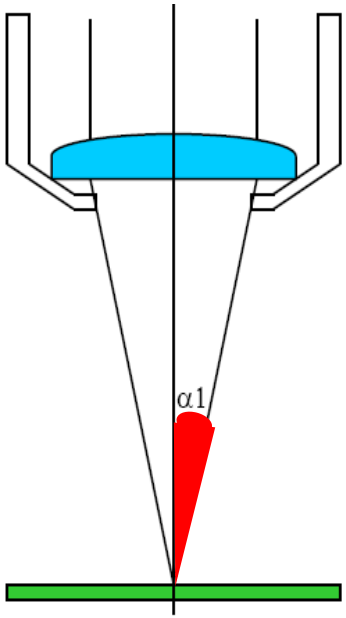
Autofluorescence (AF) and solutions

- AF---green channel worse; red or far red channel better
- AF Fixatives—wash with 0.1% borohydride
- AF quenchers: i.e. Sudan black
- Unmixing: Confocal, Nuance

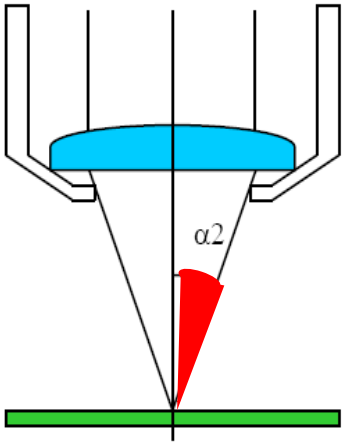
Understanding objectives—NA & immersion medium

Numerical Aperture

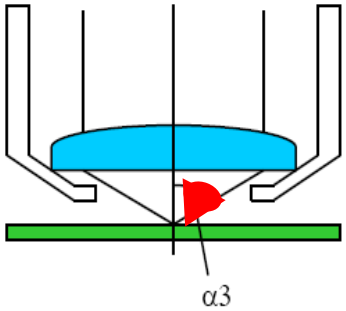
$$NA = n \sin \alpha$$



10x **Dry (1.00)**
NA 0.42



60x **Water (1.33)**
NA 1.20



60x **Oil (1.52)**
NA 1.42

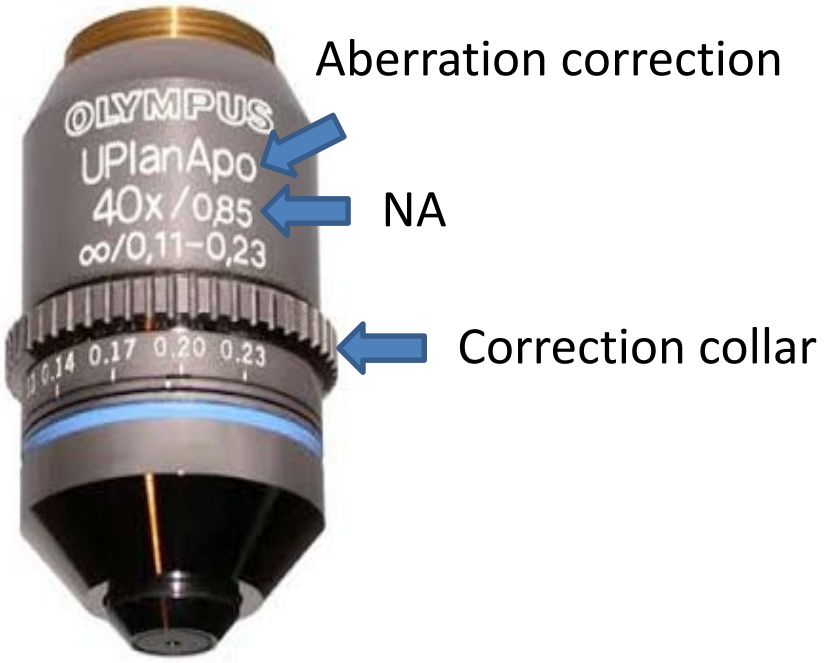
https://www.microscopyu.com/microscopy-basics/introduction-to-microscope-objectives

Understanding objectives—objective correction factors

Objective Specification	Spherical Aberration	Chromatic Aberration	Field Curvature
Achromat		1-2 Colors	No
Plan Achromat	1 Color	2 Colors	Yes
Fluorite	2-3 Colors	2-3 Colors	No
Plan Fluorite	3-4 Colors	2-4 Colors	Yes
Plan Apochromat	3-4 Colors	4-5 Colors	Yes

A good quality objective means…

- High NA lens
- Plan Apochromatic lens
- Correction collar



Designing IF experiments

- **Negative controls—false positive**
  - No staining at all—Endogenous background
  - Staining controls—No 1St Ab, non-specific staining
    2nd Ab only, Isotype 1st Ab, absorption ctrl, knockout

- **Positive controls—verify protocol and 1st Ab**
  - Known sample
  - Others: Immunoblots, single staining

- **Interpreting IF--**staining pattern

Take-home note

Olympus VS 120 scanner

Thank you!