Nanoscopy

Marcel, Wolfgang

Biomolecular Photonics, Bielefeld University

SoSe 2015

Times, dates, exercises, language, etc.

Lecture

- Lecture each Mo, 2 p.m., D3-203
- Do you prefer German or English? (The slides will be English)
- Slides (most of them) will be available online

Exercises

- Demonstration of microscopes, lab techniques, image processing
- You can make suggestions!
- Planned bi-weekly on We, 2 p.m., but we will mix that up

History and working principle of microscopes

Microscopy

An instrument to see structures too small to be resolved by the naked eye.

Microscopes boost what we know about biology: Discovery of cells

From eye-glasses and magnification-glasses (1500), early ideas by Galileo (1625), to Abbe and Zeiss (1866).

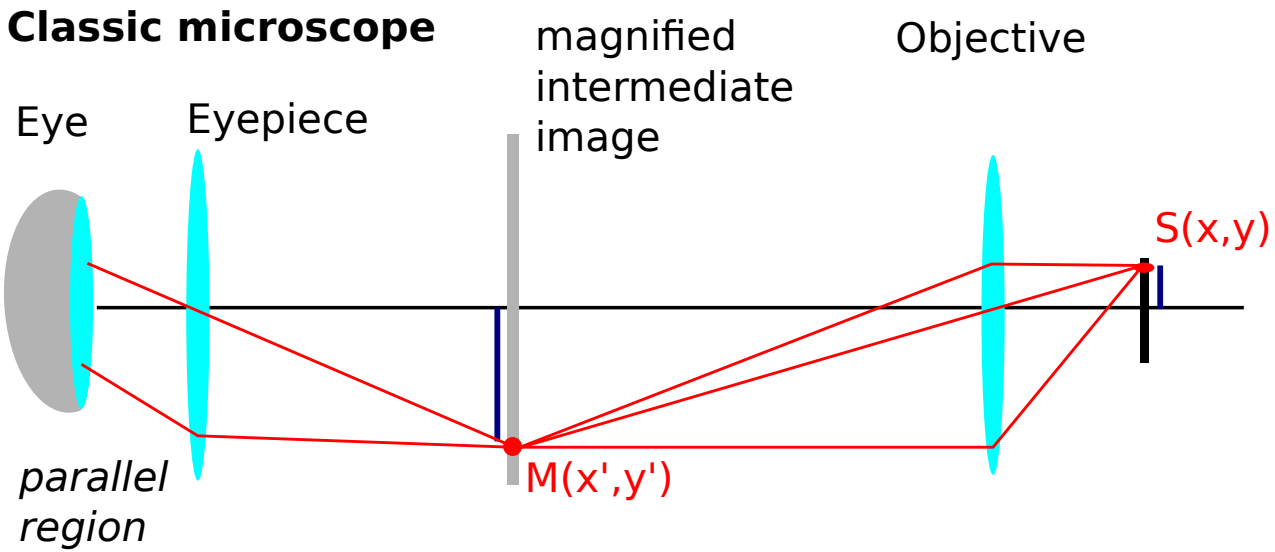
Abbe calculated how to build lenses and microscopes.

Microscope Abbe

Modern microscope

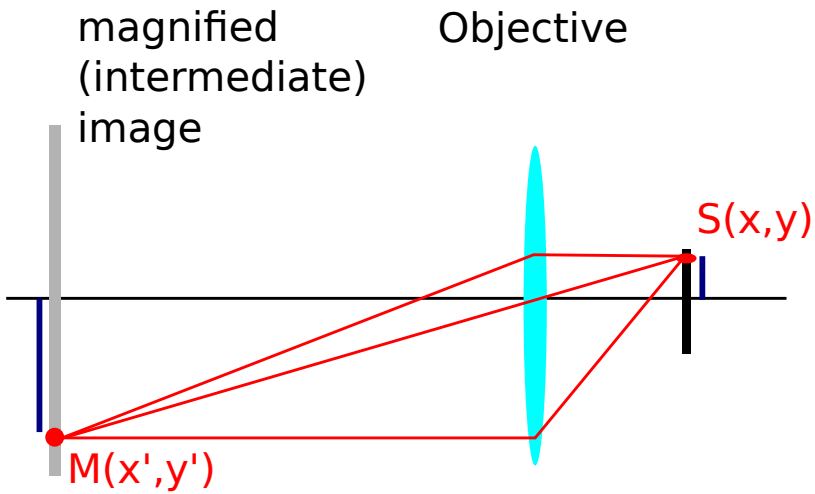
Wikimedia / Microscope

Working principle



- **Objective**: create an intermediate, magnified image of a sample
  Simple versions consist of only one lens, but better objectives use multiple lenses for different corrections.
- **Eyepiece**: Projects the intermediate image into the eye.
  Works like a magnification glass, the image plane appears at infinite distance.

Light detection

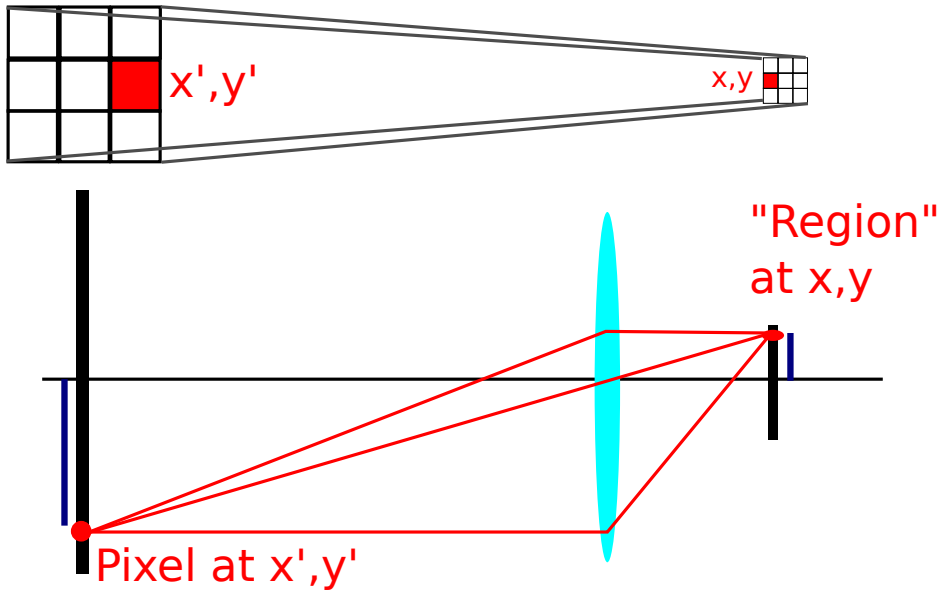


- The intermediate image is real, i.e.: A screen placed there will show the magnified sample
- Place a **photo-sensitive sensor** (CCD, CMOS) there.

Magnification and pixel size

$$M(x', y') = I \cdot S(x, y)$$

- Magnification f links $x' = f \cdot x$ and $y' = f \cdot y$. Ideally f neither depends on x, y nor on wavelength. Good microscopes are close enough to that ideal.
- Camera: Array of photo detectors (pixels), typical size $d' = 50 \ldots 150\,\mu\mathrm{m}$.
- **Get used to**: *effective pixel size*, i.e. pixel size projected on sample: With $d' = 75\,\mu\mathrm{m}$ and $f = 60\times$, $d = 125\,\mathrm{nm}$.



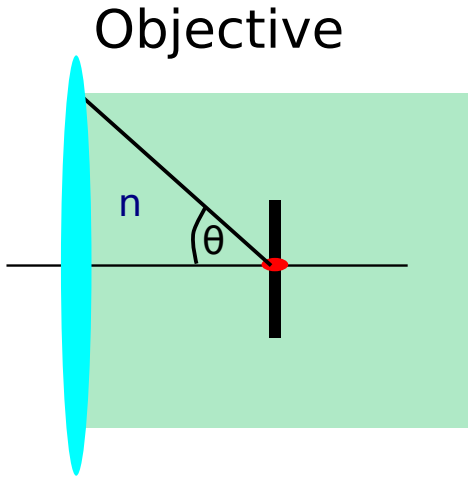
Camera pixel: Rectangular area collecting photons, thus integrating intensity. Maps to a (usually and ideally) regtangular area on the sample.

Resolution of a microscope

The Abbe diffraction limit

$$d = \frac{\lambda}{2\,n\sin(\theta)} = \frac{\lambda}{2\,\mathrm{NA}}$$

Modern objectives reach 1.5 NA with immersion media.

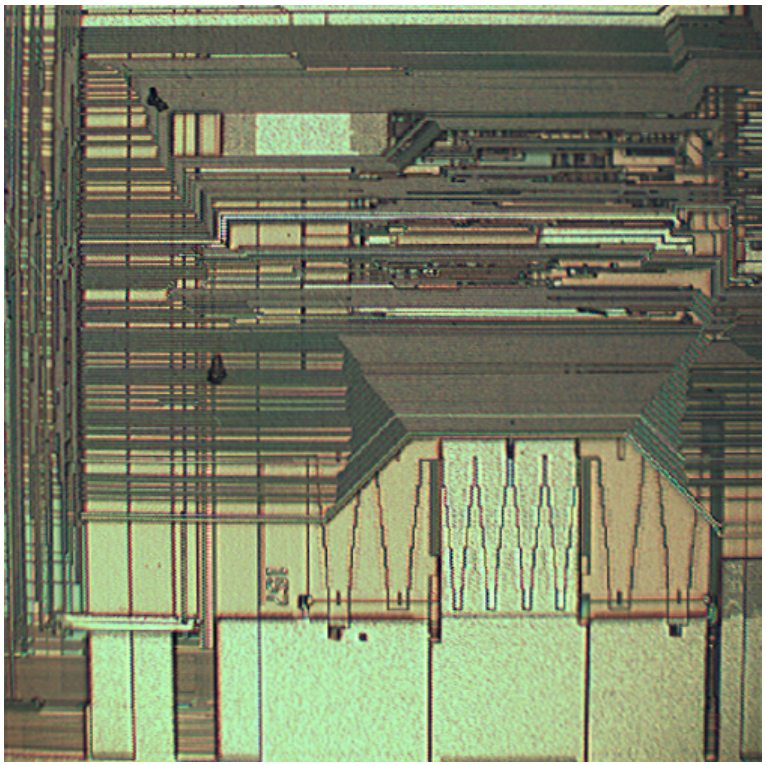


Resolution vs. contrast

- Resolution is more complex, especially for 3-dimensional samples / axially
  $\rightarrow$ Point-spread functions, missing axial cone, etc.
- Contrast is also important, and closely linked to fluorescence microscopy

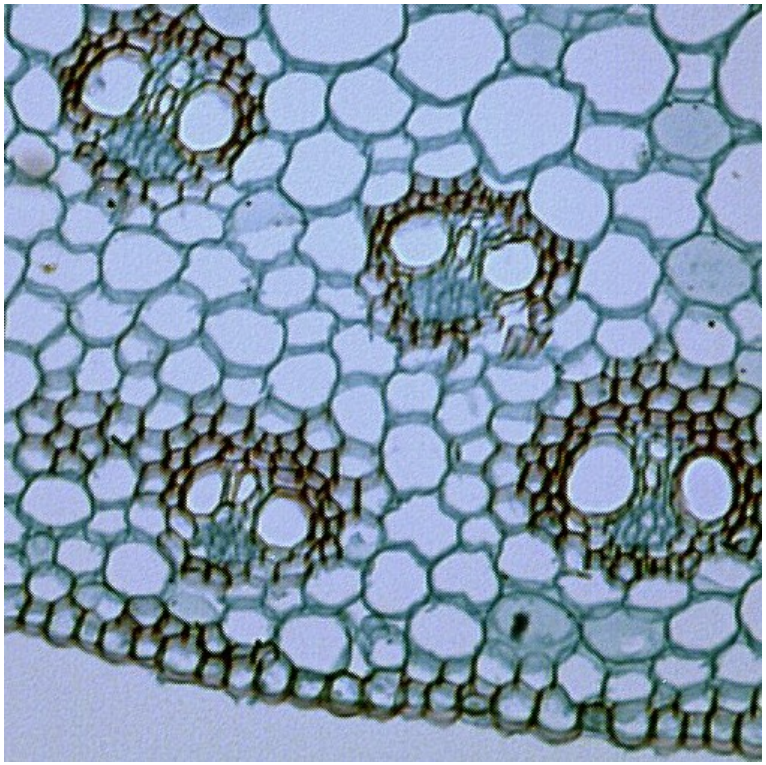
Microscopy of surfaces or 3D structures

Look at light scattered from a surface. Image formation like taking a photo...



Wikimedia / 80486 chip

...but biological samples are rarely flat, reflective surfaces. Light shines *through* the sample.

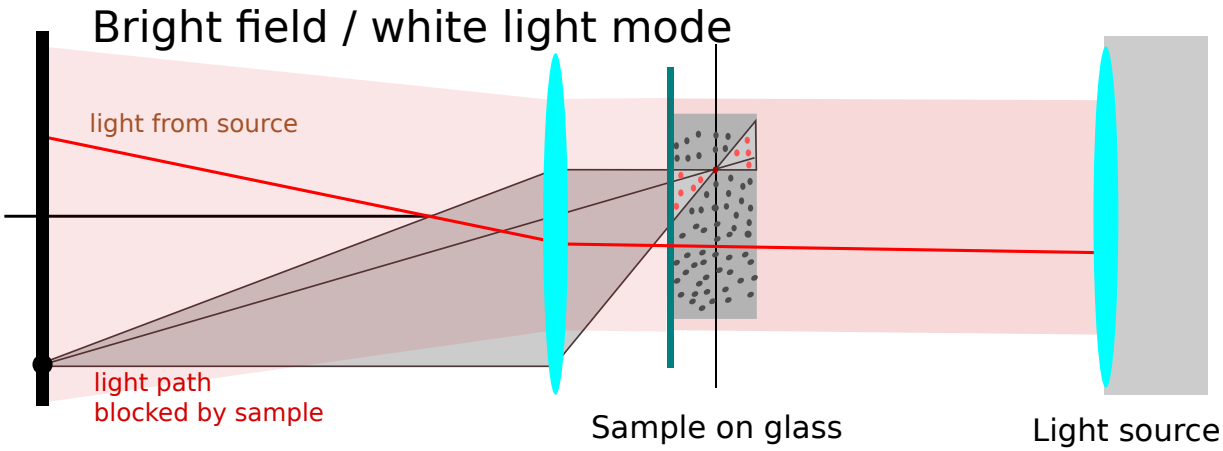


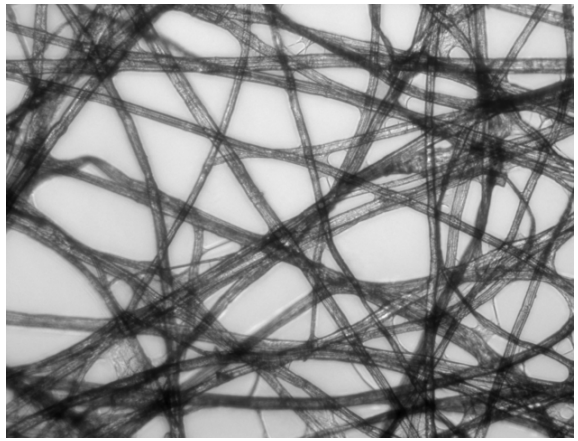
Wikimedia / Vascular tissue

Classic illumination modes

Reflection, Scattering, Absorbtion...
How do we generate an image?

Illumination/"classic": Bright field / white light mode

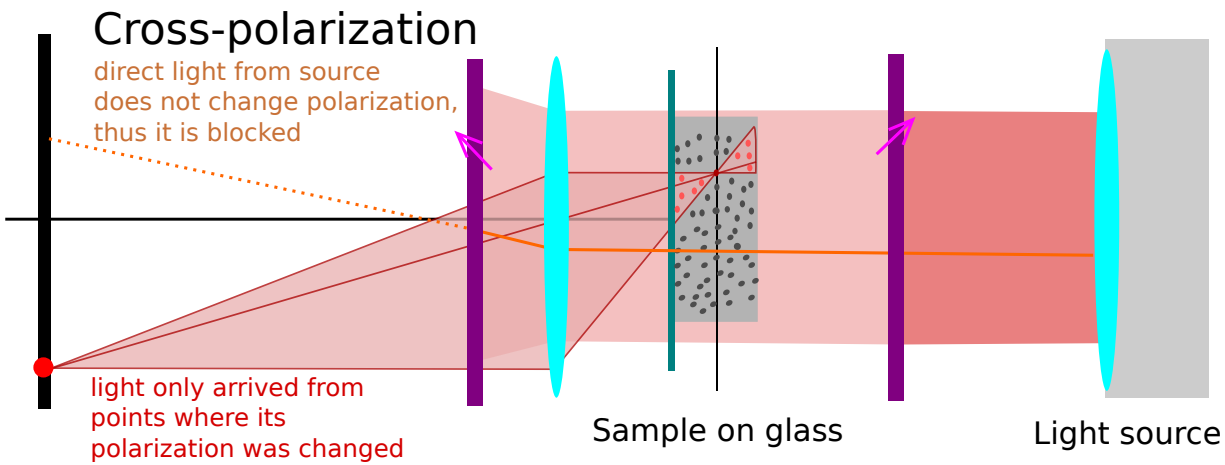


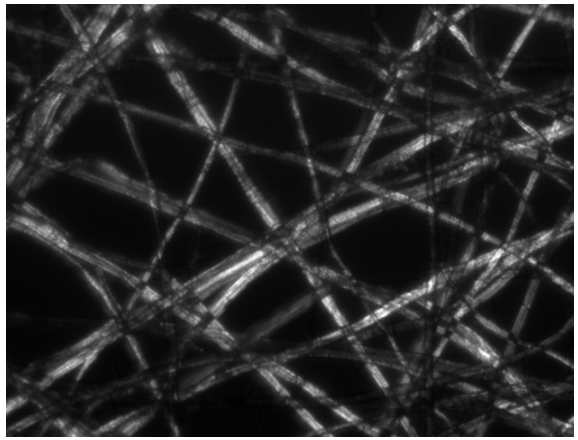


Wikimedia/Bright Field

- Arguably the oldest and most standard mode
- Light goes through the sample: "Durchlicht"
- Contrast by absorbing and scattering light
- Optimized by using **Köhler illumination**
- Used at (almost) every microscope e.g. to quickly align a new sample

Illumination/"classic": Cross-polarization

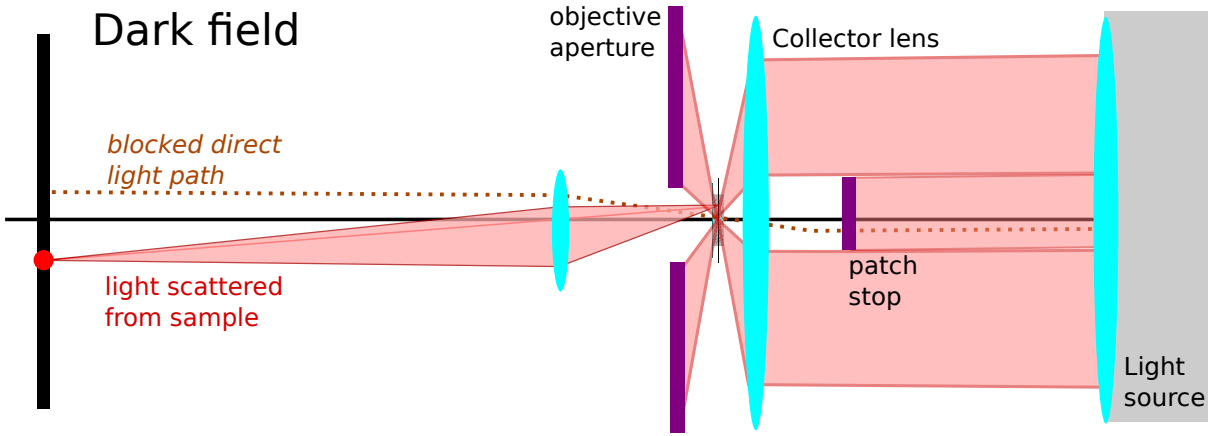


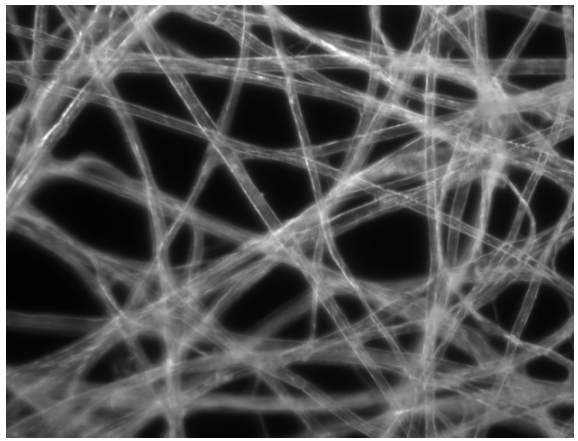


Wikimedia/Cross-polarized

- Easy extension to bright field: Two polarization filters
- Polarized light enters the sample:
  - If it does not interact, it is blocked by a second filter
  - If it interacts, and changes polarization, it can pass the second filter
- Visible: Structures that change the polarization of light

Illumination/"classic": Dark field



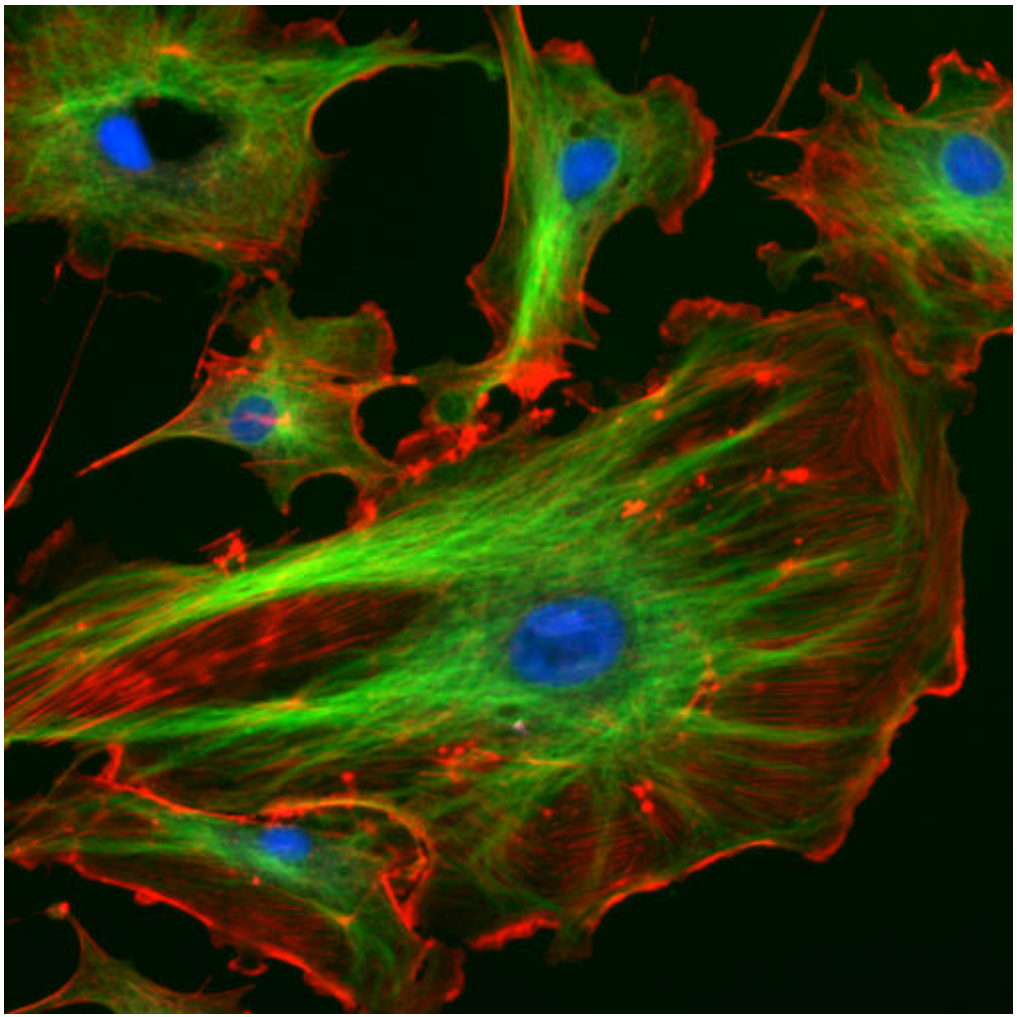


Wikimedia/Dark Field

- Align illumination optics and block central beams
- Light enters sample under a steep angle:
  - If it does not scatter, it exists under the same angle. Thus it is blocked by the imaging objectives aperture
  - If it scatters, some will leave under a flat angle, able to pass the aperture
- Visible: All scattering structures
- This can be extended to measure (almost only) phase-shifting structures.

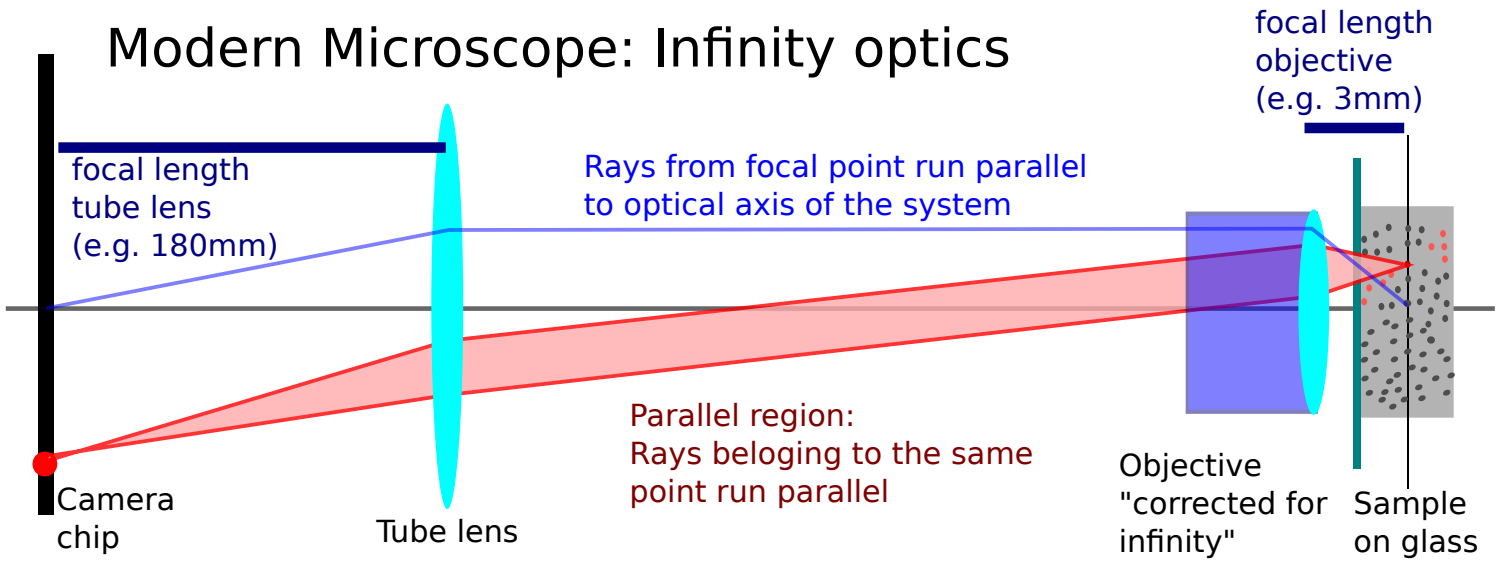
The problem with "classic" illumination

For most biological samples, scattering and absorption does not yield much information. The following would not be possible:



Wikimedia/Fluorescence Cell

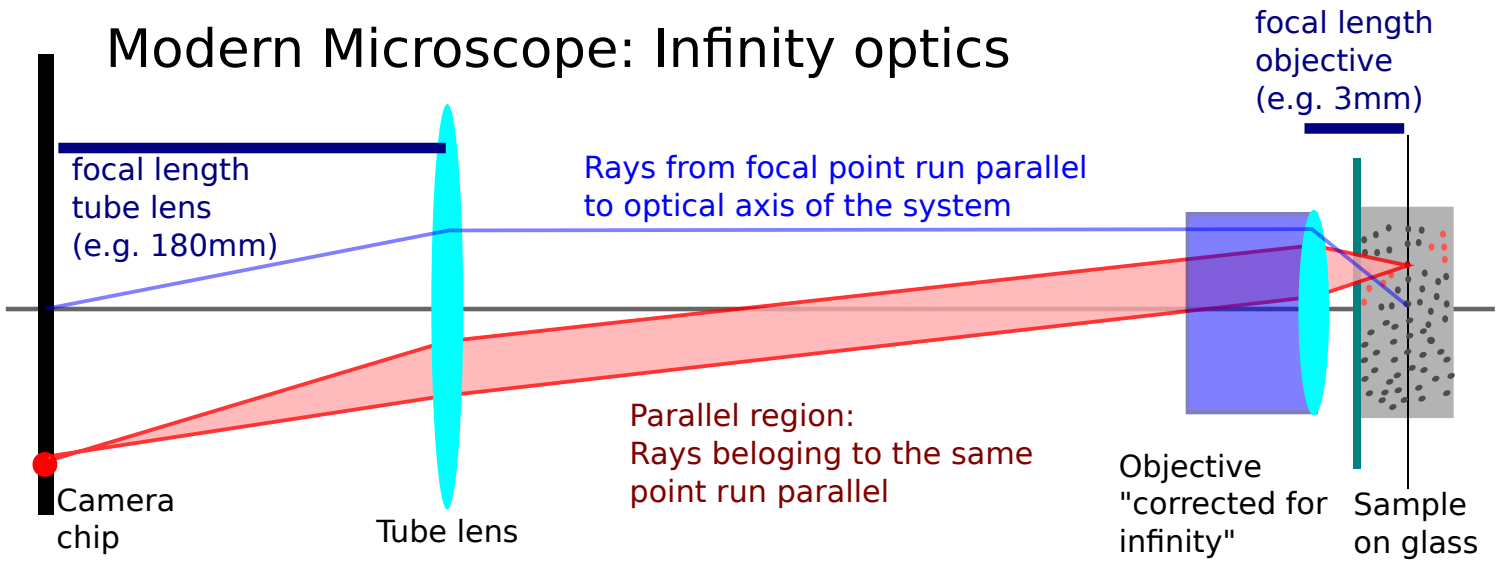
Excursion: Infinity optics



$$\frac{1}{f} = \frac{1}{b} + \frac{1}{g}$$

- Set $g = f$, then $b = \infty$, image forms "in infinity"
- Set $b = f$, then $g = \infty$, objects at infinity distance are now in focus
  That one actually works on it own, take a landscape photo with $g >> f$
- For an imaging system, plug two of these ($b_1 = f_1, g_1 = \infty$, $b_2 = \infty, g_2 = f_2$) together
- Advantage: "Parallel region" for light manipulation (filters, mirrors)

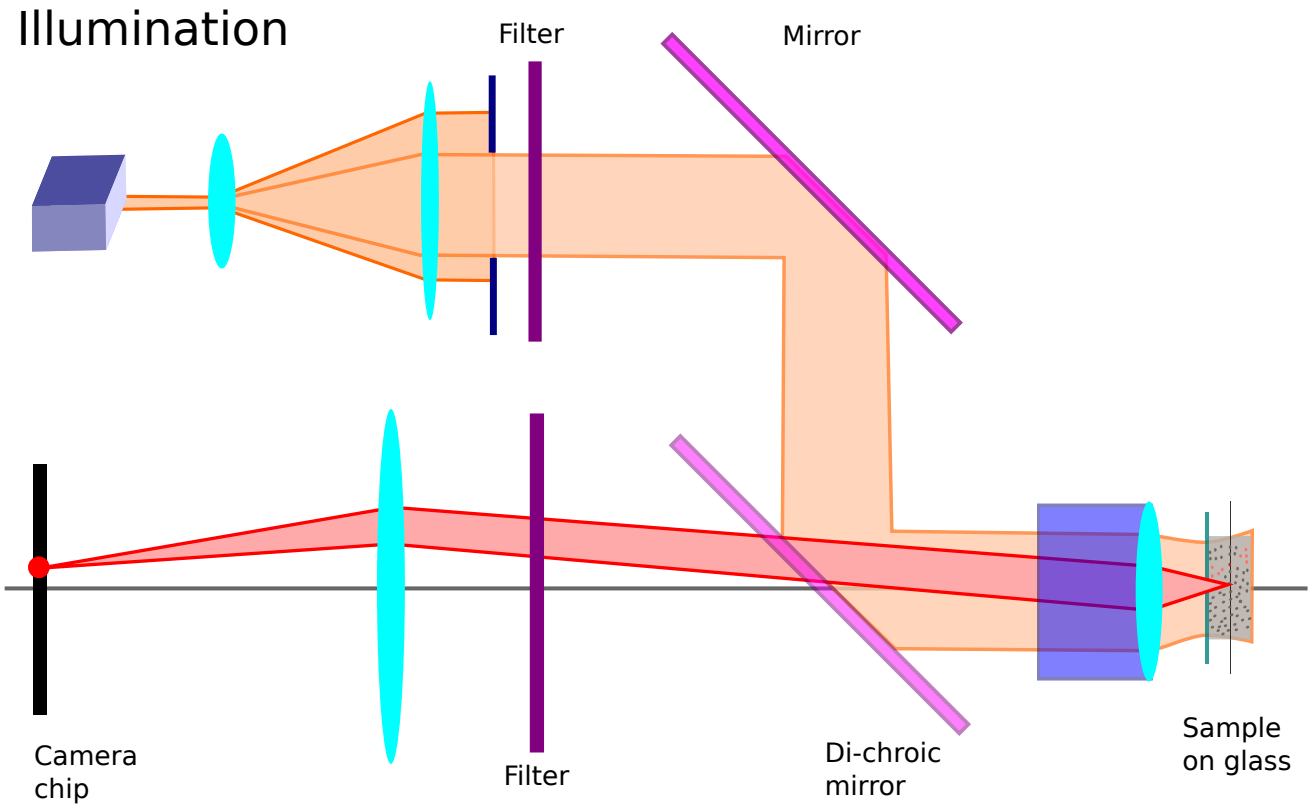
Excursion: Infinity objectives



- **Magnification** becomes easy: $\frac{f_1}{f_2}$, here $\frac{180\,\mathrm{mm}}{3\,\mathrm{mm}} = 60\times$
- "Infinity objectives" are no magical, physics-defying things
  - They are a bunch of (well made, well adjusted, expensive) lenses
  - "Infinity" means: Lens corrections (coating, calculations) are optimized for infinity focus applications (at more than one wavelength)
  - Often they state a magnification ($60\times$), not a focal length (3 mm). In these cases, there is a (manufacturer-dependent) standard tube lens (e.g. 180 mm) that gives that magnification.

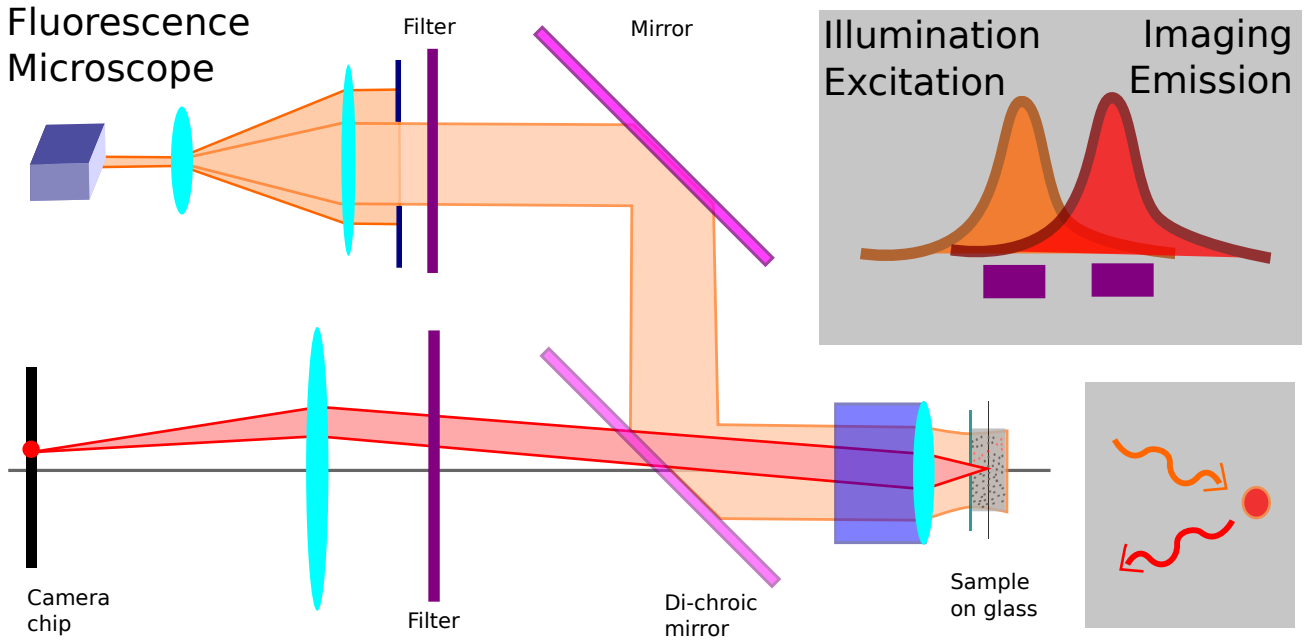
Introducing the fluorescence microscope

Widefield flourescent microscopy: Light



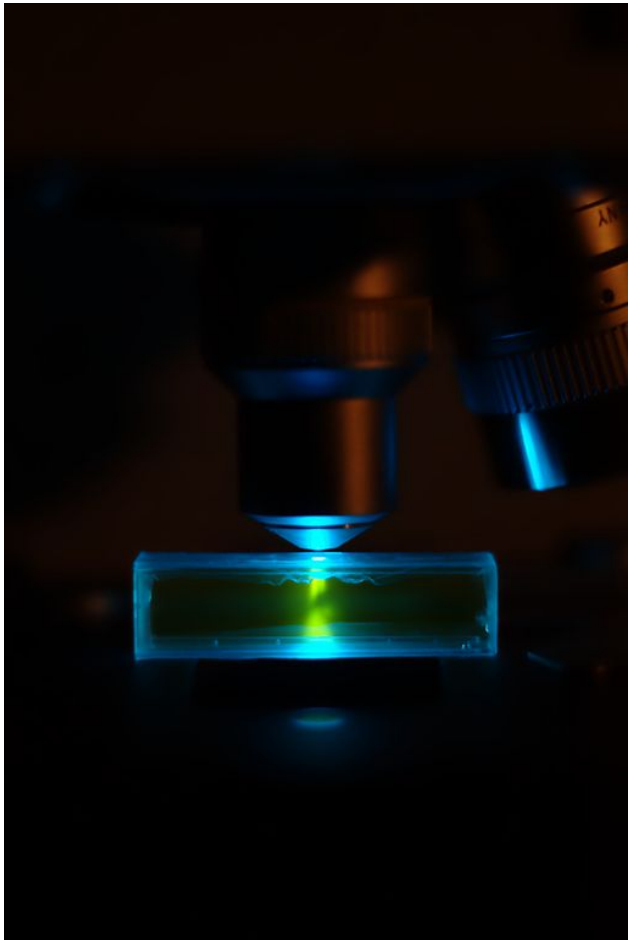
- Illumination and imaging share a light path through the same objective
- Easier adjustment, no restriction on sample thickness
- Would this setup do bright field? Dark field?

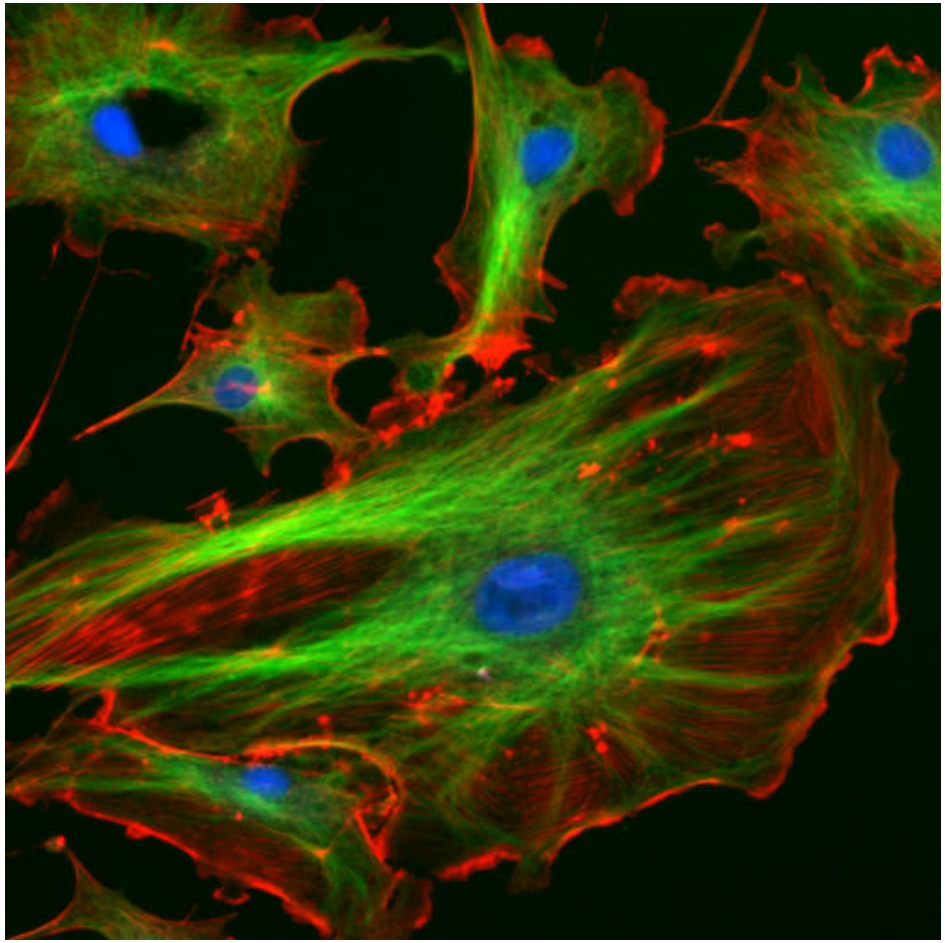
Widefield flourescent microscopy



- Fluorophores capture photon, hold it for some nanoseconds, emit it at a longer wavelength (in any direction, with any polarization)
- Fluorophores have an excitation and an emission spectrum.
- Lamp/Laser filter: Illuminate the excitation spectrum
- Camera filter: Image the emission spectrum
- Ideally: Little to no overlap (with good filters)

Widefield flourescent microscopy: Images





Wikimedia/Fluorescence Sample Wikimedia/Fluorescence Cell

- Blue illumination, green fluorescence
- Endothelial cells

nuclei blue by DAPI, microtubules green by antibody-FITC, actin red by phalloidin-TRITC

What this lecture will cover

Interdisciplinary field

- **Biology** asks the questions
- **Biology** provides fluorescence labeling strategies
- **Chemnistry** researches more and better fluorescence dyes
- **Physics** builds complex microscopes
- **Engeneering** creates lenses, light detectors, laser sources
- **Computer science** implements image analysis algorithms
- **Math** inspires these algorithms

Topics: Basics and super-resolution

Basics

- Fluorophores
  - Physics: Stokes-shift
  - Biology: Labeling techniques
- Crash-course: Cells
- Microscope components
  - Optical path: Lenses, objectives, filters, ...
  - Light detection: CCDs, CMOS, PMTs, APDs
  - Light sources: Lamps, LEDs, Lasers
- Resolution and contrast
  - Point-spread functions
  - Axial resolution
  - Background, Noise
  - Optics in Fourier space: OTFs

Advanced techniques

- **Structured illumination**
  $\approx \sqrt{2} - 2\times$ beyond Abbe, fast
  - TIRF illumination
  - Confocal scanning
  - SR-SIM: Working principle and reconstruction algorithm
  - 3D-(SR)-SIM / Optical sectioning
- **Localization microscopy**
  $\approx 2 - 50$ nm, slow(er)
  - Deterministic, e.g. STED, RESOLFT
  - Stochastic, e.g. STORM, PALM
  - Reconstruction algorithms for stochastic localization microscopy