Advanced light microscopy

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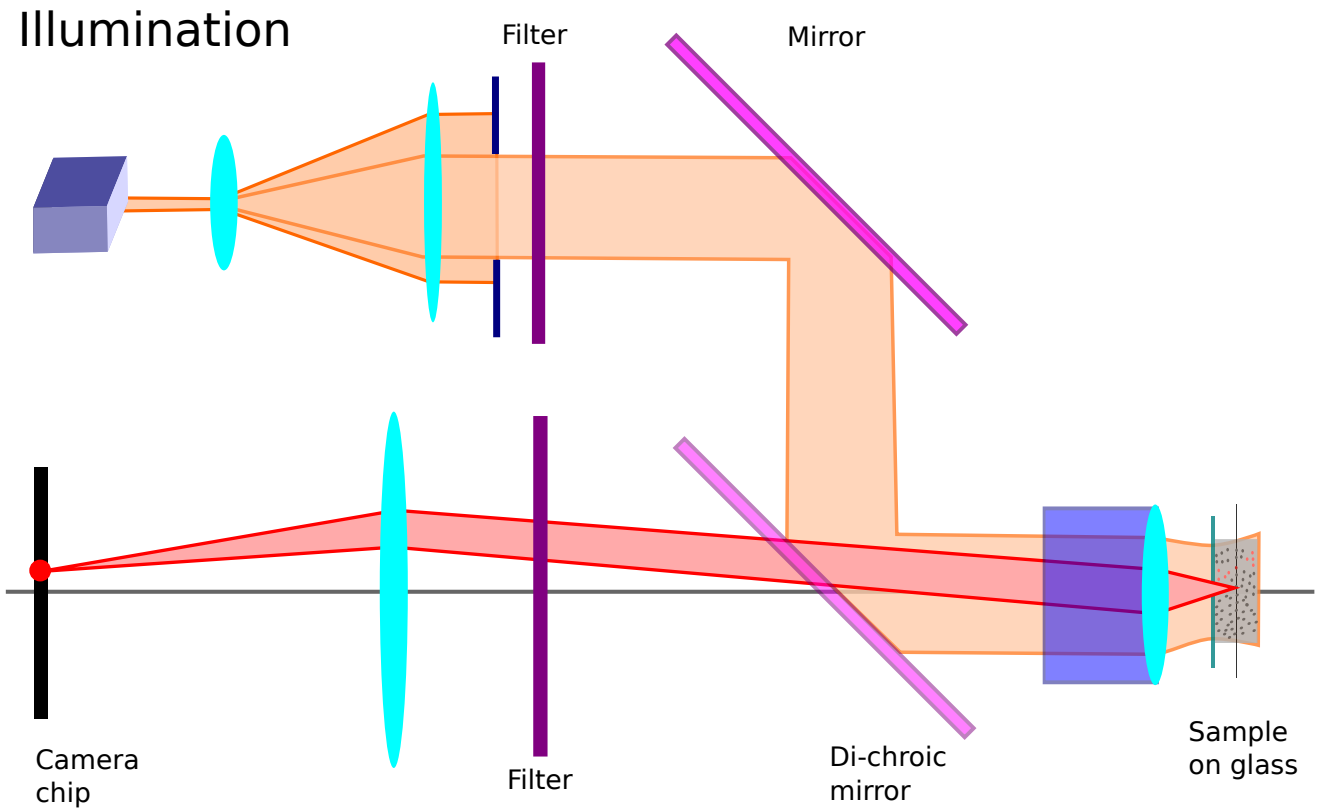
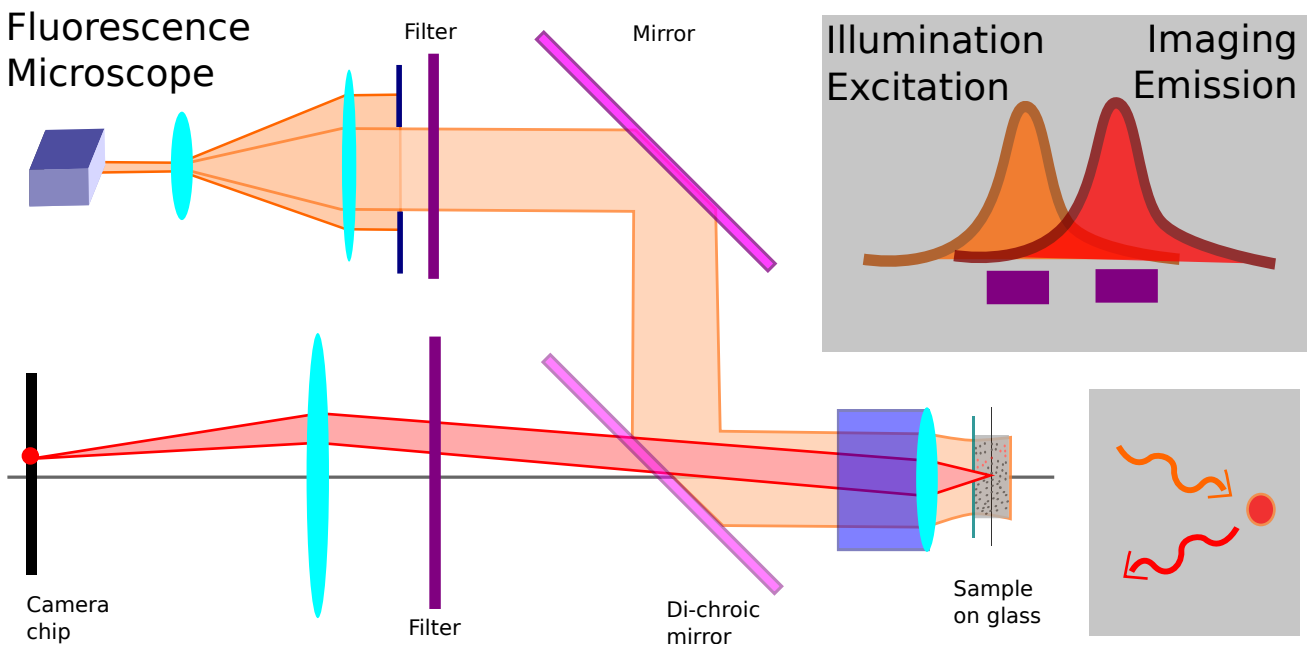
Fluorescence microscope setup



- Illumination and imaging share a light path through the same objective
- Easier adjustment, no restriction on sample thickness
- Need a way to separate illumination light from sample response:
  In practice: By wavelength

Fluorophoes



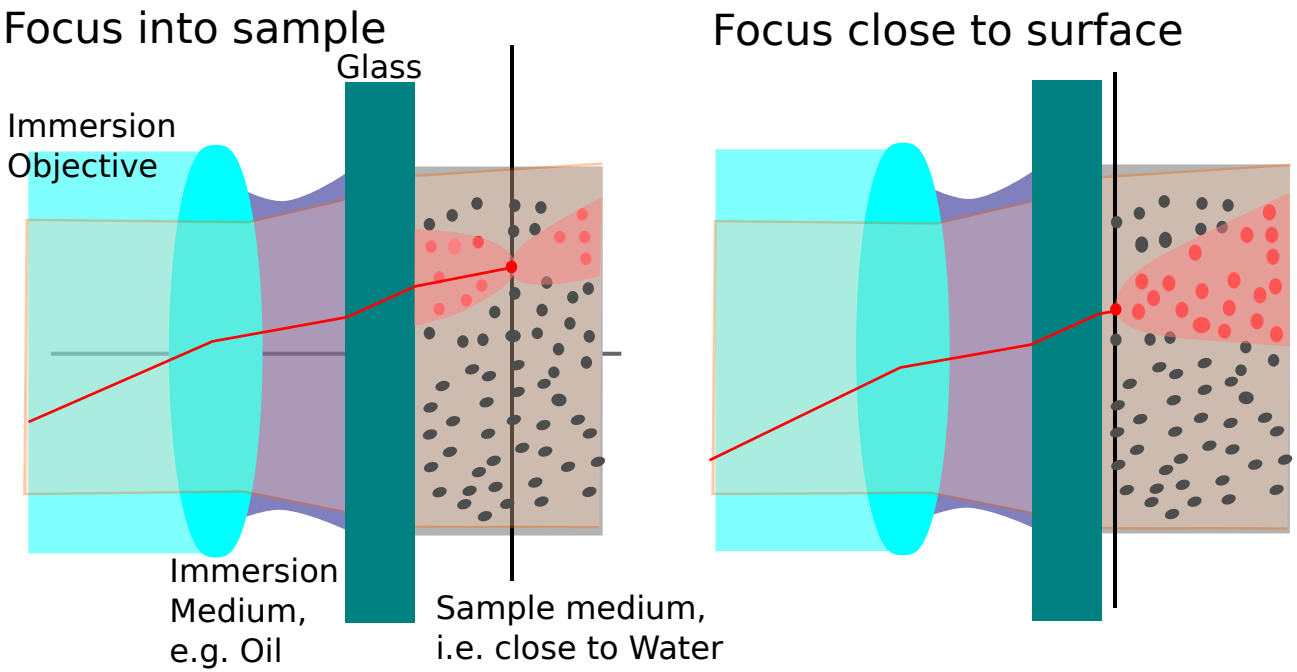
- Capture a photon, hold it for some nanoseconds, emit it at a longer wavelength (in any direction, with any polarization)
- Have an excitation and an emission spectrum (separated via filters)
- **Allow to mark specific structures**
- Outlook: There are other processes that shift wavelength. . . measuring these is much more involved.

Mathematical description

$$M(x,y) = \int_{S_z} \mathrm{PSF}(z) * (I(x,y,z) \cdot S(x,y,z))\,\mathrm{d}z$$

- I is the illuminating light, here still uniform
- S as the sample response, now *fluorophore density*, in principle a collection of single molecule delta functions.
- The system will lose (a lot of) photons. For counting purposes, we would have to quantify that.

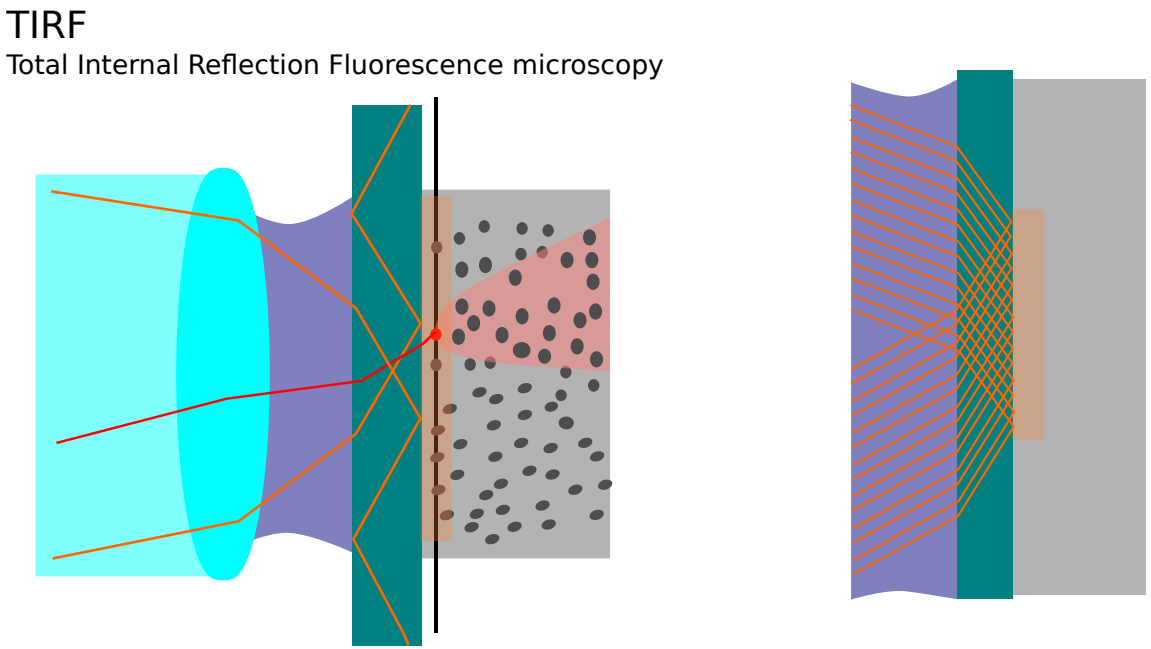
Axial resolution / contrast improvement



$$M(x,y) = \int_{S_z} \mathrm{PSF}(z) * (I(x,y,z) \cdot S(x,y,z))\,\mathrm{d}z$$

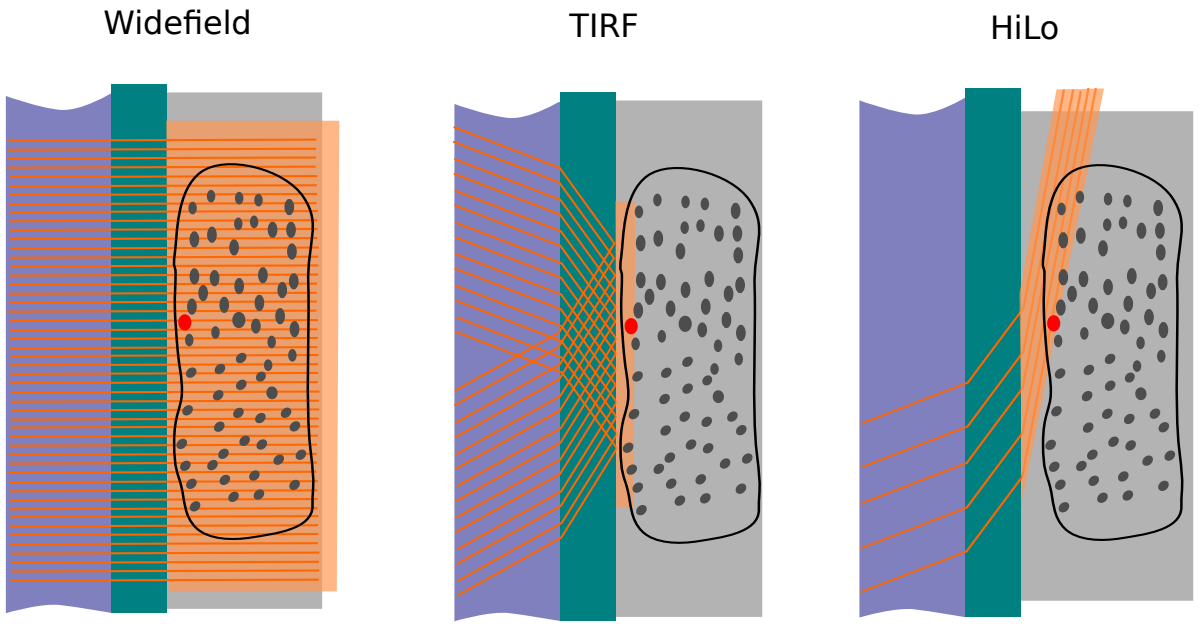
- Problem for axial resolution and background:
  The integral $\int_{S_z}$, where the PSF gets for $z \neq 0$.
- Idea: change the modulation $I(x,y,z)$ so that $I \approx 0$ for $z \neq 0$.
- Multiple methods to approximate that. Arguably the easiest: TIRF and HiLo.

TIRF



- Typical sample: Refraction index close to water.
  Therefore, total reflection between glass and sample.
- Evanescent wave, so $I(z) = I_0 \cdot e^{-\alpha \cdot z}$ with I_0 at the sample / glass interface.
- Enough light for a focal plane up to a few hundred nanometers from glass (that the drawback).

Three light modes



- **Widefield** illuminates all the sample uniformly. Free to chose focal plane, but prone to background contributions.
- **TIRF** lets the illumination intensity drop exponentially with distance to the glass / sample interface. Only usable for structures close to glass, but very little background
- **HiLo** illumination angle close to TIRF, thus a sheet of light passes through the sample. Often a good compromise, but quantifying the light intensity takes some work.

Outlook on super-resolution

$$M_{I,\kappa}(x,y) = \int_{S_z} \mathrm{PSF}(z) * (I_I(x,y,z) \cdot S(x,y,z,\kappa))\,\mathrm{d}z$$

PSF cannot be overcome.
But other factors can be influences, with two mayor approaches:

- **Influence the illumination**: Use multiple sets I of $I_I(x,y,z)$, where $I_I(x,y,z)$ varies along x,y,z. If now $M_I(x,y)$ and $I_I(x,y,z)$ is known, solve for $S(x,y,z)$.
  First steps are TIRF, confocal scanning, state of the art: **Structured illumination microscopy**
- **Influence the sample response**: Add some *property* κ to the sample, so its response to illumination can change. This can be switching the fluorophore (STED) or a stochastic process (STROM, dSTROM), allowing for **localisation microscopy**.
- Finally, both approaches can even be combined.

Demonstration

Remaining time of this lecture:
Hands-on demonstration of TIRF on our microscope