Nanoscopy

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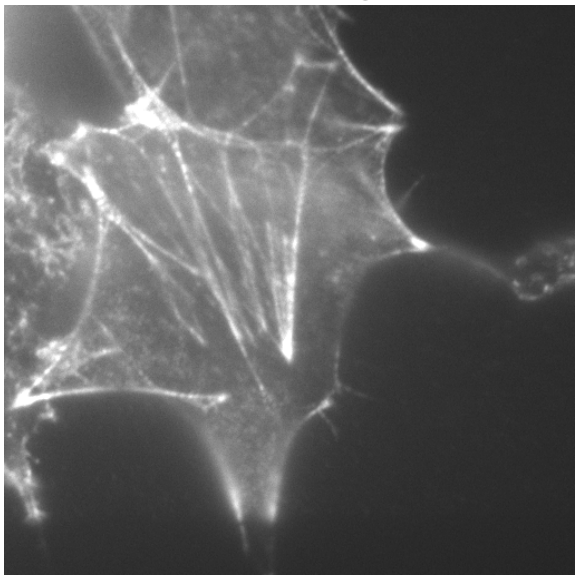
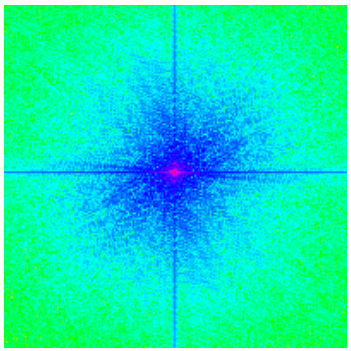
1. Recapitulation
   - Background
   - Fluorescence microscopy
2. Illumination modes, (con)focal scanning
   - TIRF
   - Confocal scanning
3. Summary and Outlook

Background: Examples

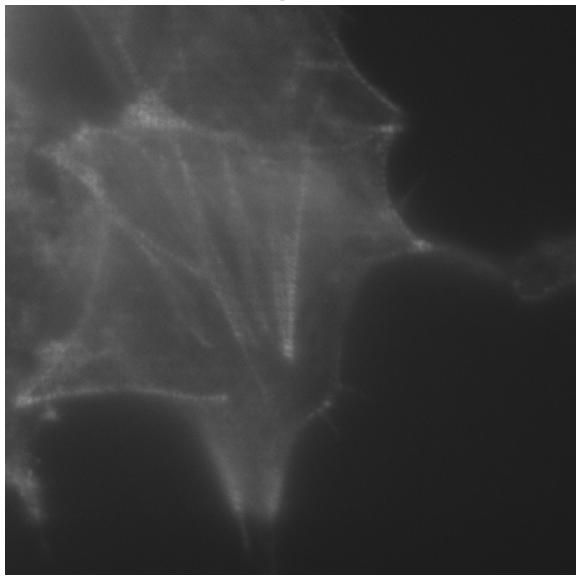
Background are photons picked up from out-of-focus z-positions on the sample.

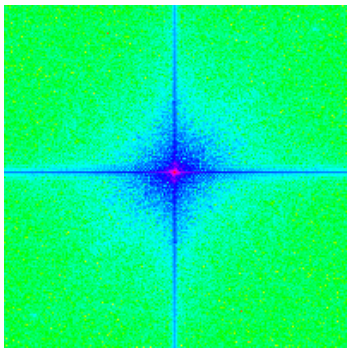
Full Image



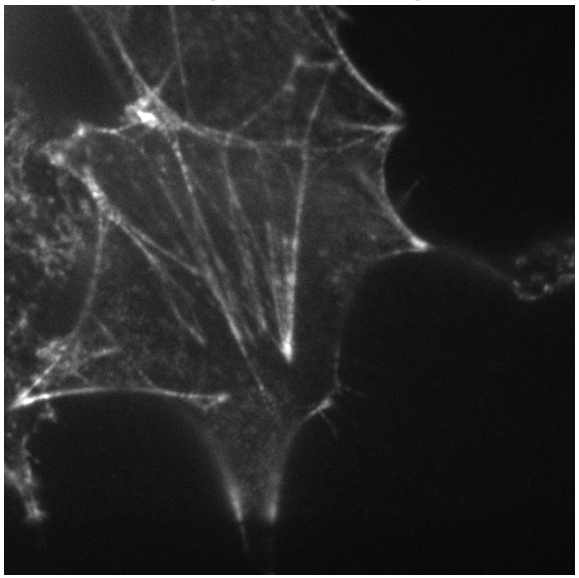


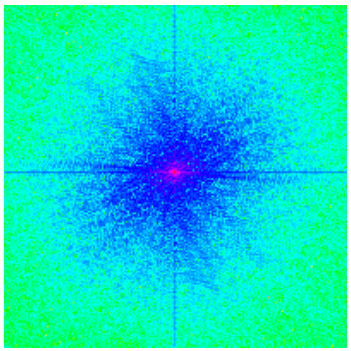
Background





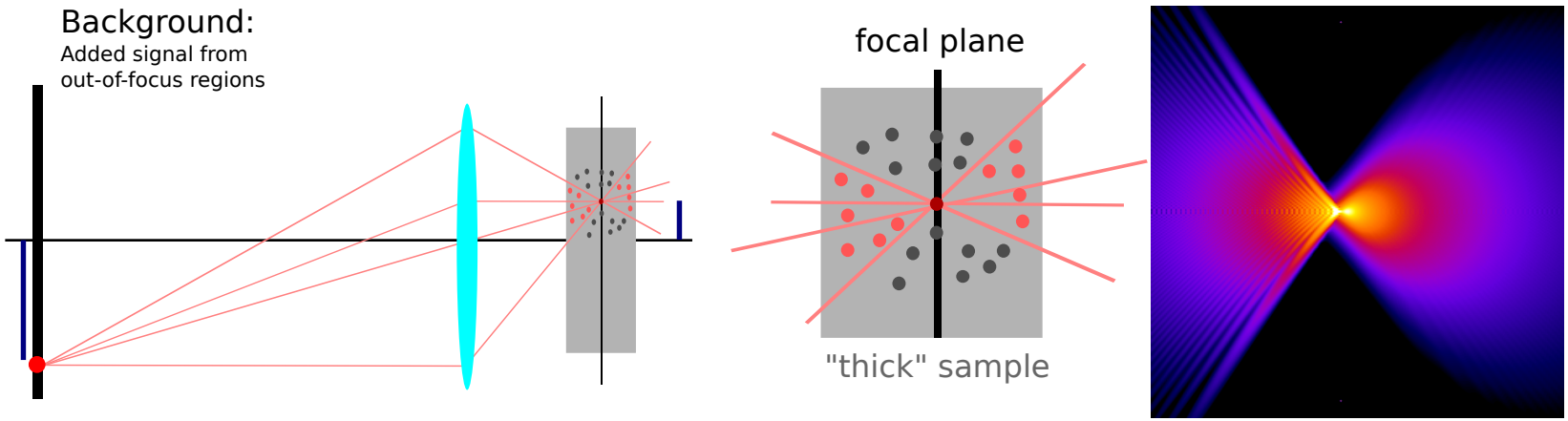
Full Image - Background





- Unwanted out-of-focus contributions
- Background scales with illumination intensity, thus SBR (signal-background-ratio) uninfluenced by more light

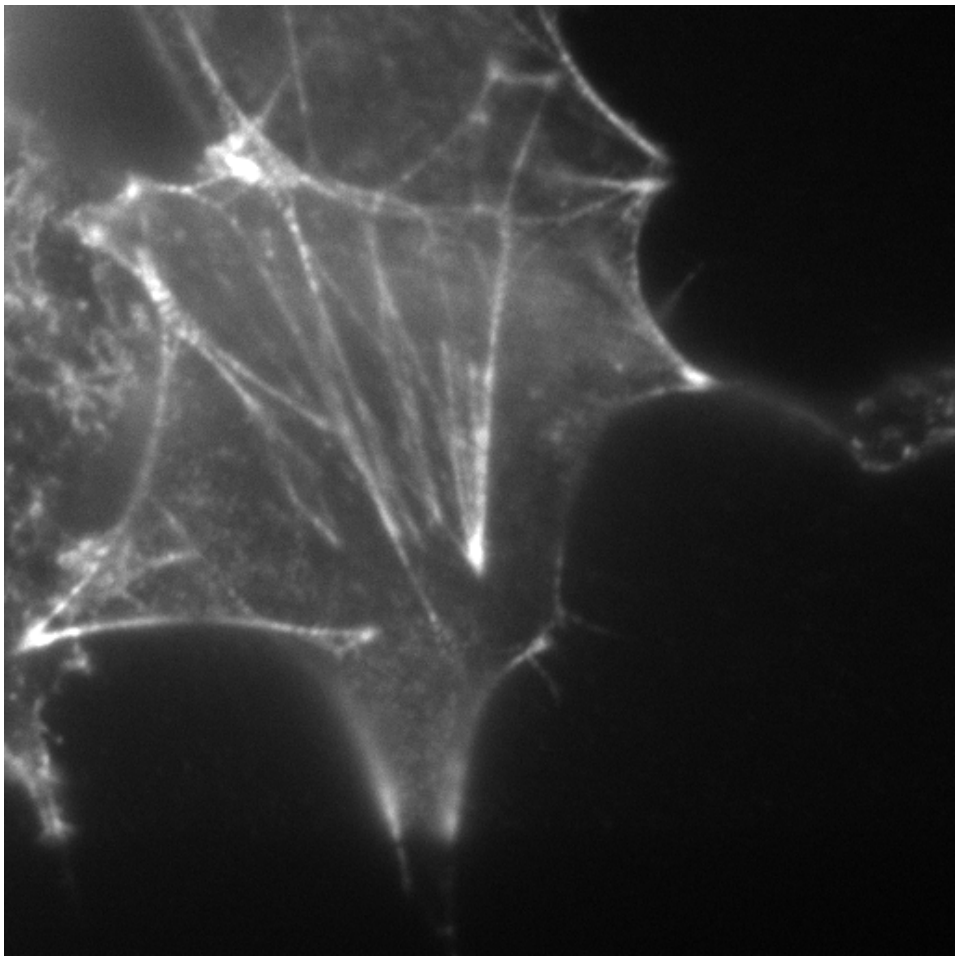
Background: PSF

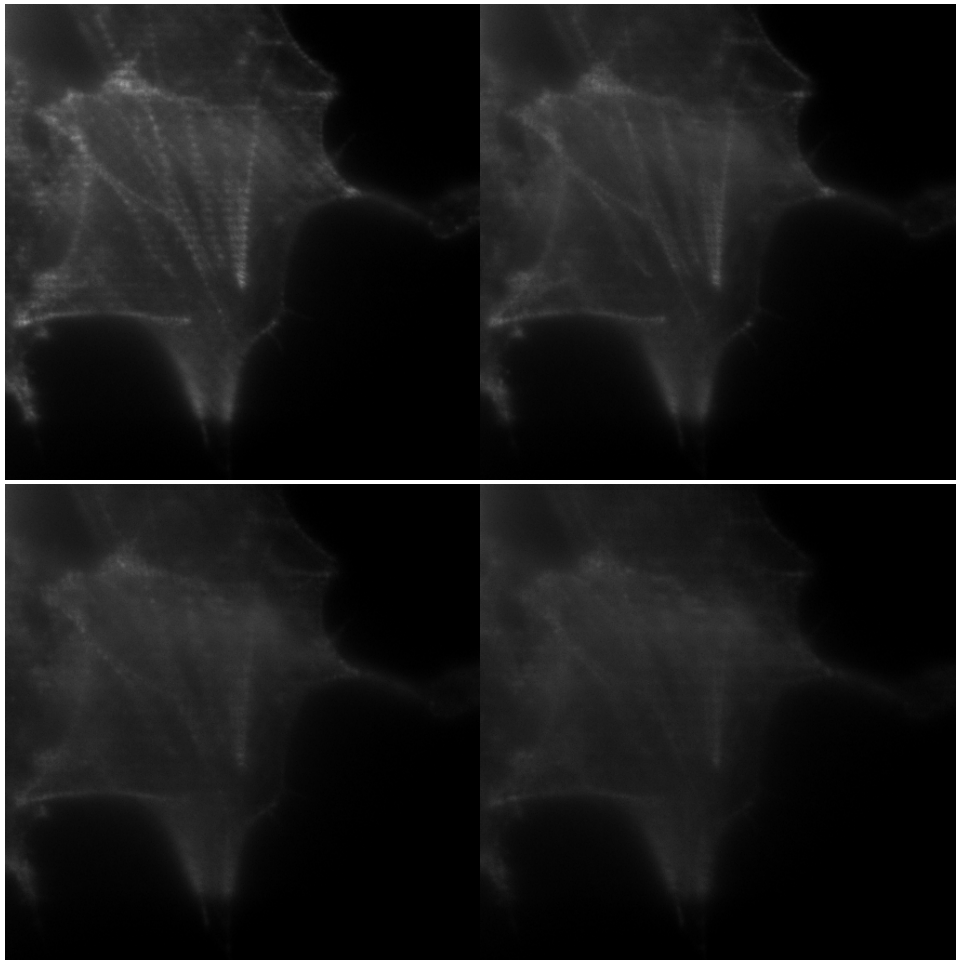


$$M(x,y) = \int_{S_z} \mathrm{PSF}(z) * (I \cdot S(x,y,z))\,\mathrm{d}z$$

- 3D PSF (and sample) to account for these contribution
- z-component generally harder to calculate, but can be measured and/or simulated.
- **Important**: Axial vs. lateral resolution

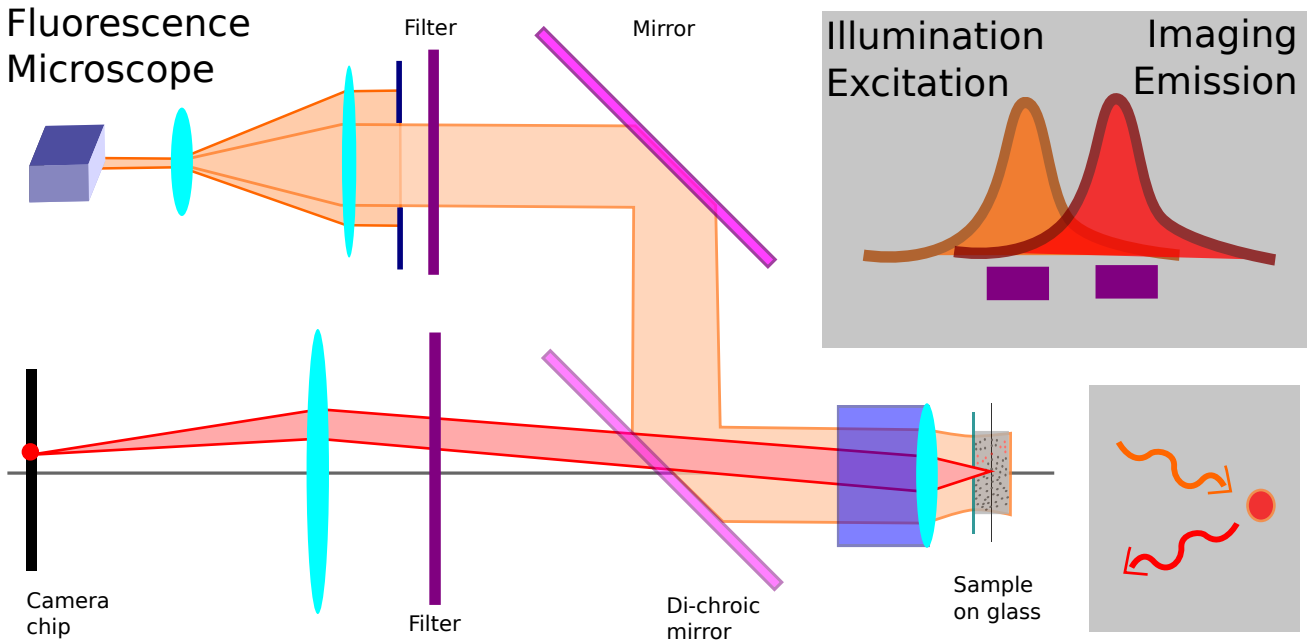
Background falloff as PSF gets wider





- Left: Widefield image, Actin-labeled cell
- Right: Contributions from different depths of out-of-focus regions

Recapitulation: Wide-field epi-fluorecence microscope

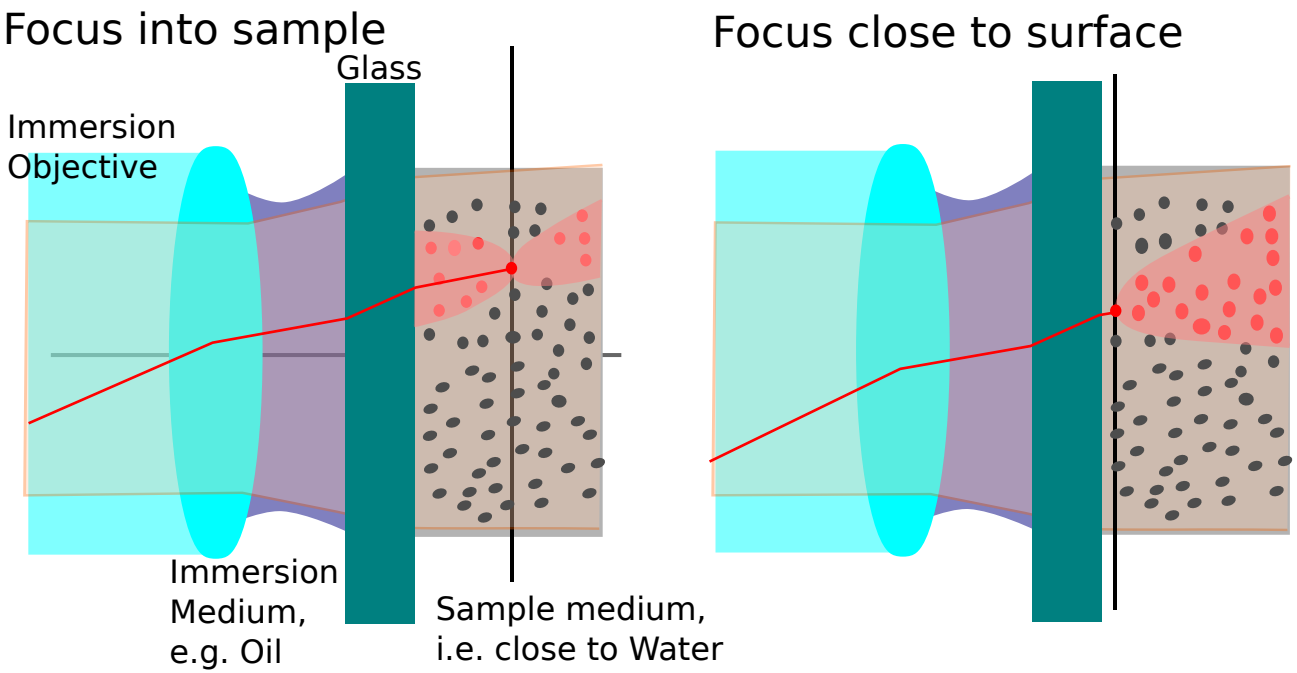


Here the image $M(x, y)$ is given by illumination intensity I and the fluorophore density / distribution S.

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

Axial resolution enhancement by changing illumination mode

Improvement: Background problem...

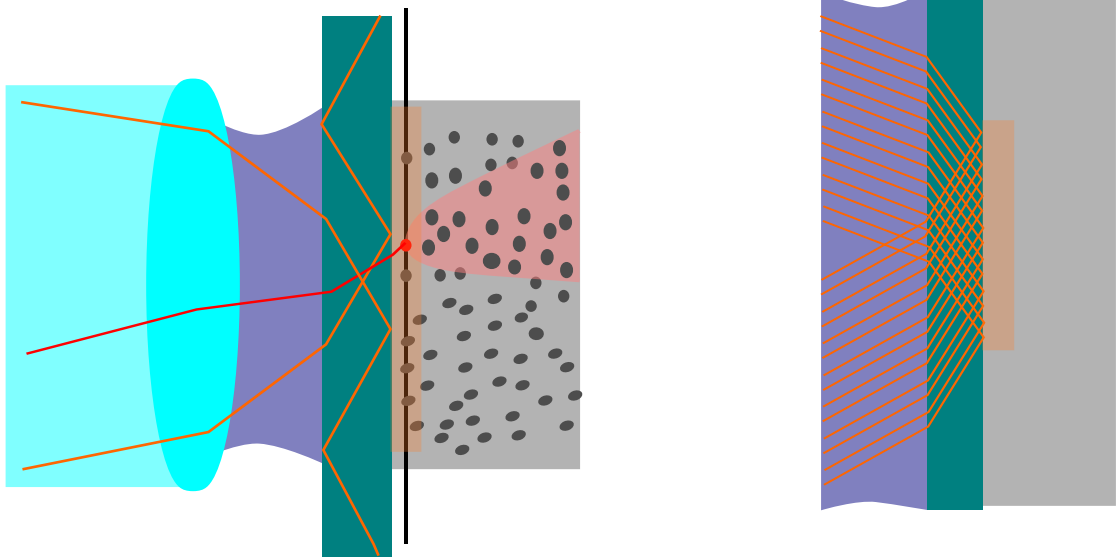


$$M(x,y) = \int_{S_z} \mathrm{PSF}(z) * (I(x,y,z) \cdot S(x,y,z))\,\mathrm{d}z$$

- Light distribution is uniform throughout sample
  $I(x,y,z) = \text{const.}$
- Idea: change I so that there is only illumination close to the focal plane
- Possible if focal plane is close to the glass

TIRF: Principle

TIRF
Total Internal Reflection Fluorescence microscopy



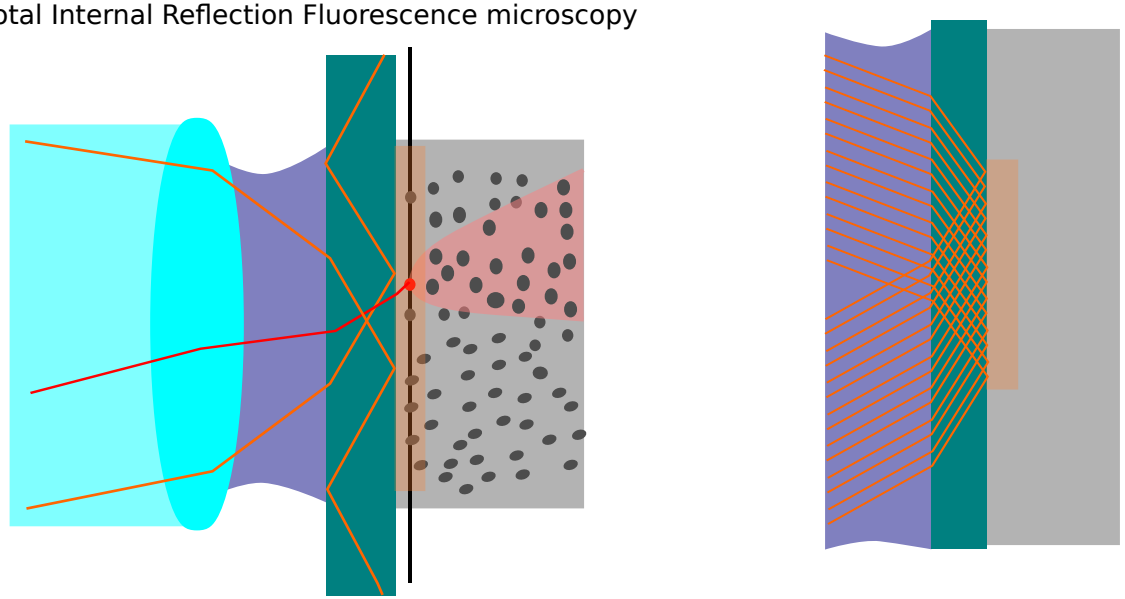
- Typical sample: Refraction index close to water.
  Therefore, total reflection between glass and sample.
- Adjust the illumination optics alignment, let light enter under a flat angle.

$$\theta = \arcsin\left(\frac{n_{\text{sample}}}{n_{\text{glass}}}\right)$$

- Why it there any light to illuminate the sample now?

TIRF: Effect

TIRF
Total Internal Reflection Fluorescence microscopy

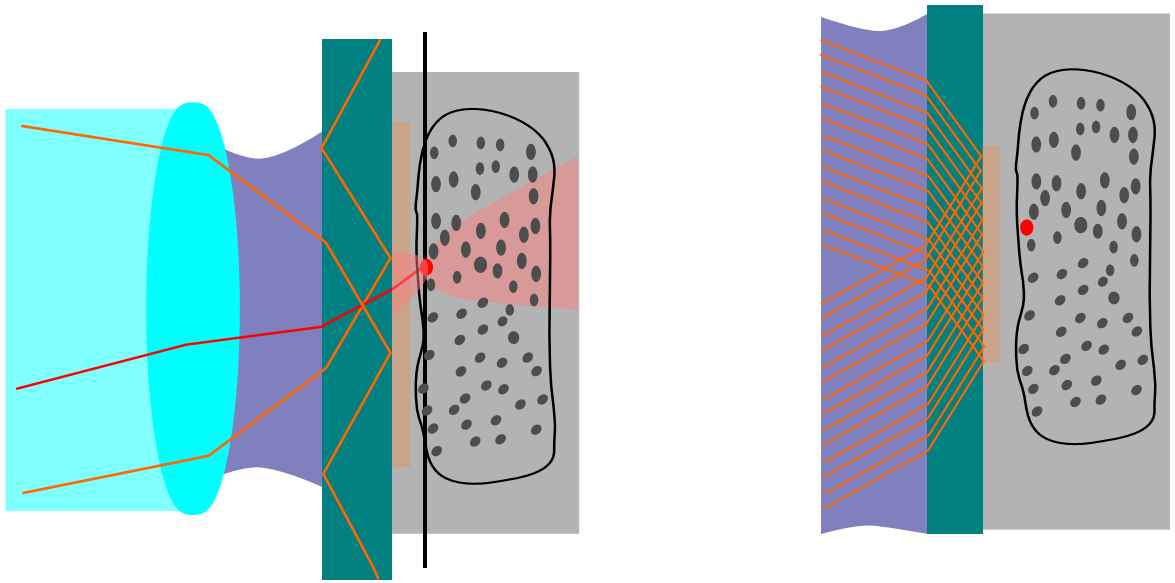


$$M(x,y) = \int_{S_z} \mathrm{PSF}(z) * (I(z) \cdot S(x,y,z))\,\mathrm{d}z \quad \text{with} \quad I(z) = I_0 \cdot e^{-\alpha \cdot z}$$

- Evanescent wave, so the intensity drops exponentially
- I_0 at the sample / glass interface where $z = 0$
- α depends on angle, refractive indices, wave length...
- Typically usable up to a few hundred nanometers from glass (that the drawback).

TIRF: Problem

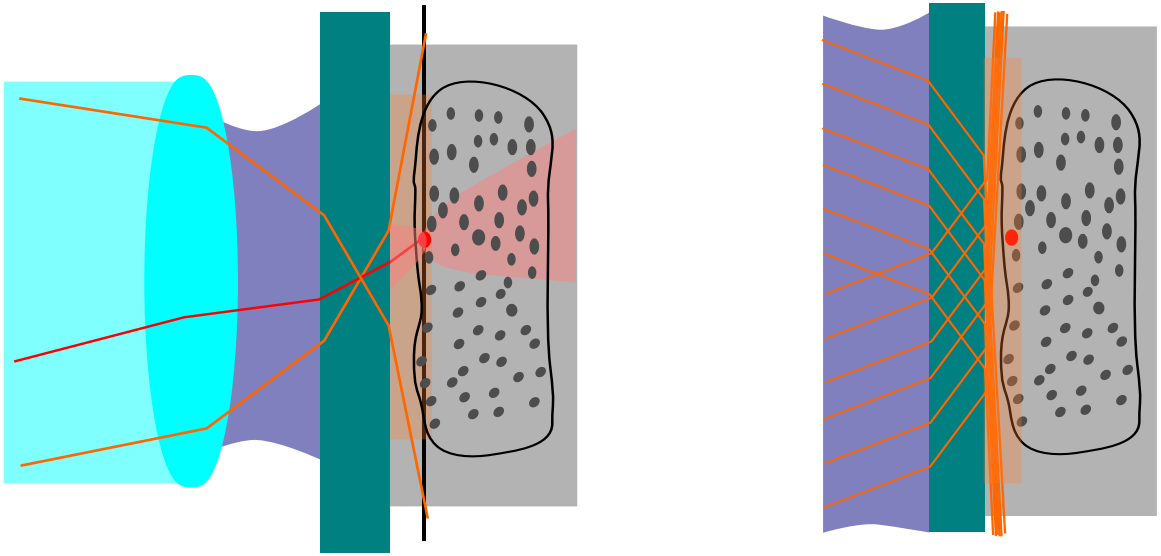
TIRF Problem: Sample not close to surface



- Fluorescent material (almost) only inside a cell
- Structure of interest somewhere near membrane
- Cell has some distance to clover slip (medium)
- Problem: Too far away for TIRF to work

Solution: work almost in TIRF

(almost) TIRF
Total Internal Reflection Fluorescence microscopy

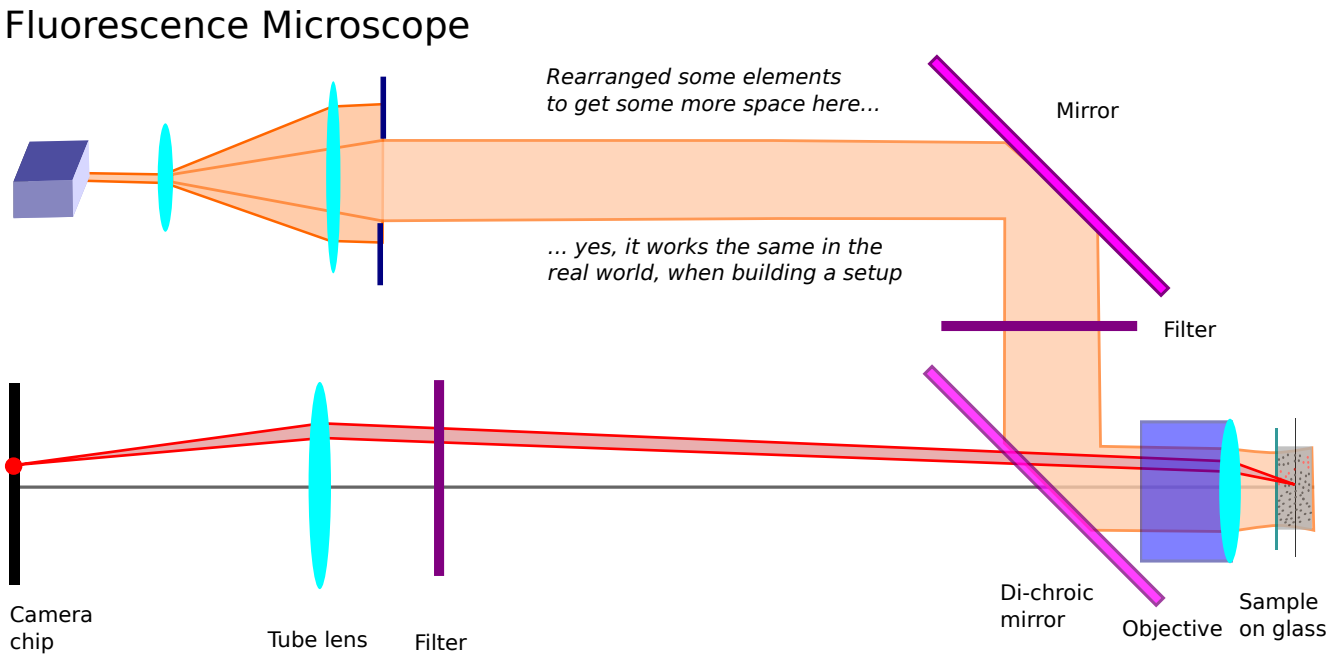


- Use a TIRF setup, which will be adjustable, find the critical angle θ by looking for TIRF, adjust some degree away from TIRF
- Light will travel through the sample under a very flat angle, which can also be adjusted
- The idea is similar to dark-field

Focal scanning

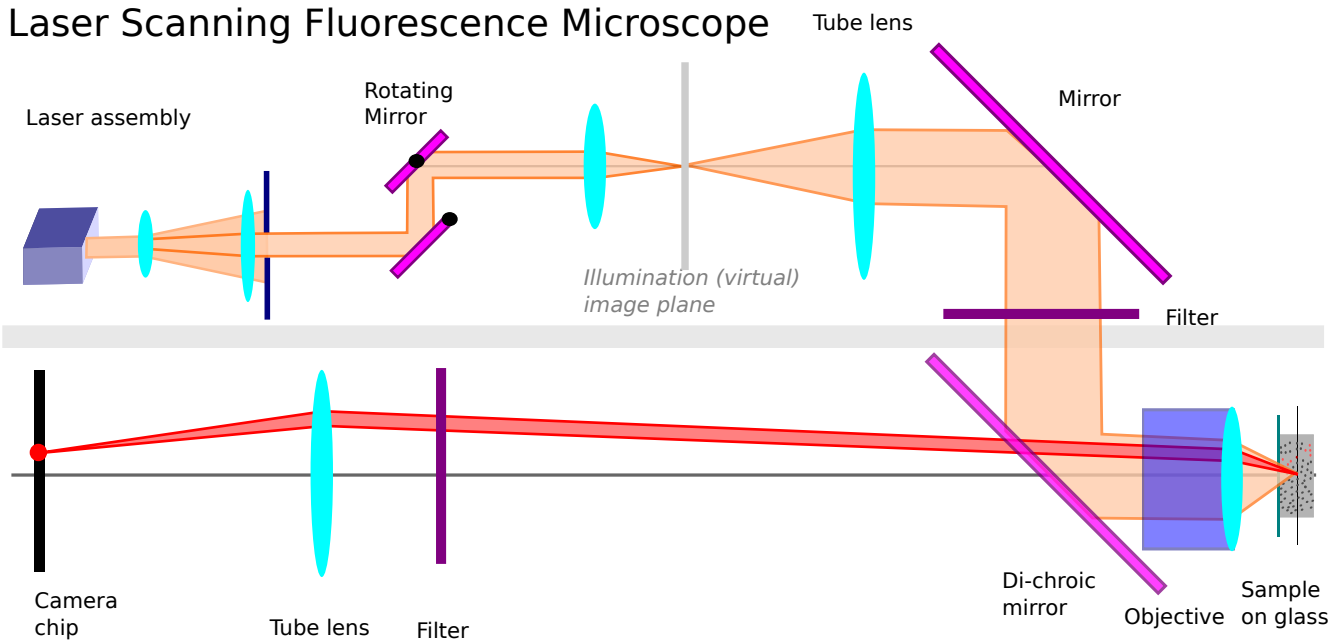
Confocal scanning: Resolution enhancement through overlapping PSFs

Focal scanning: Starting point



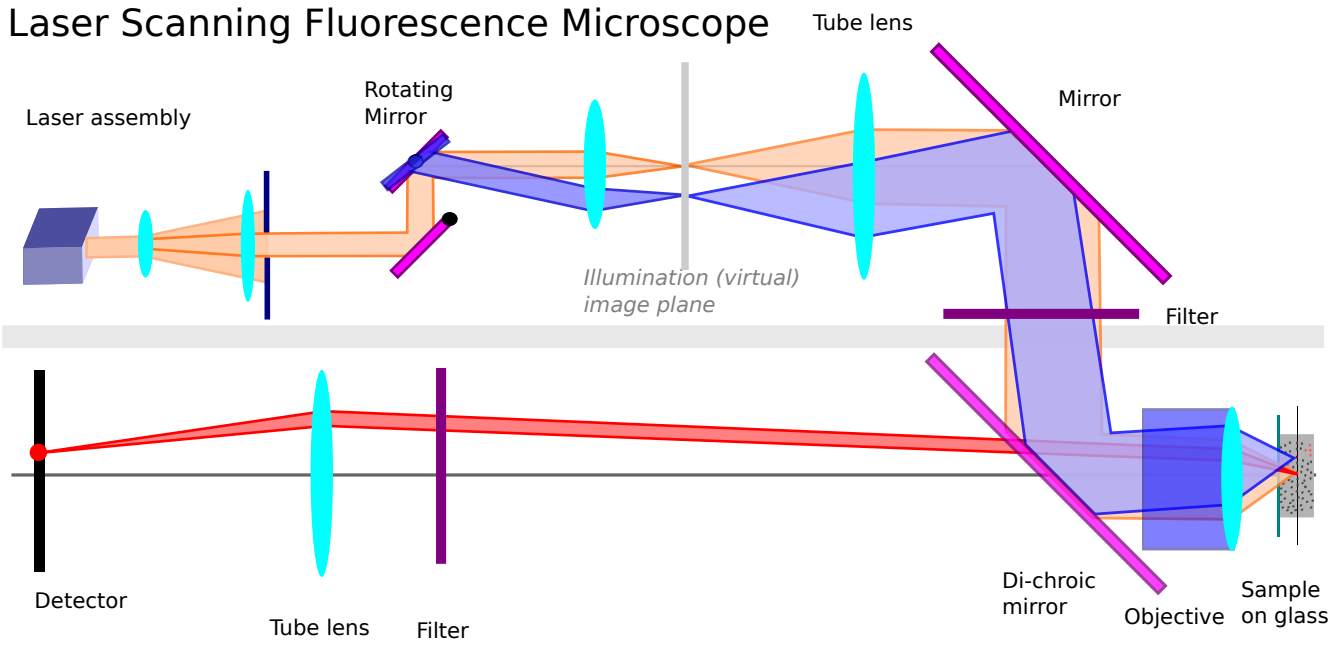
- The widefield setup, some components rearranged
- Yes, that will happen when you build a system. . .
- Note: Up to the tube lens, illumination and imaging path are the same

Focal scanning: Focussing the illumination



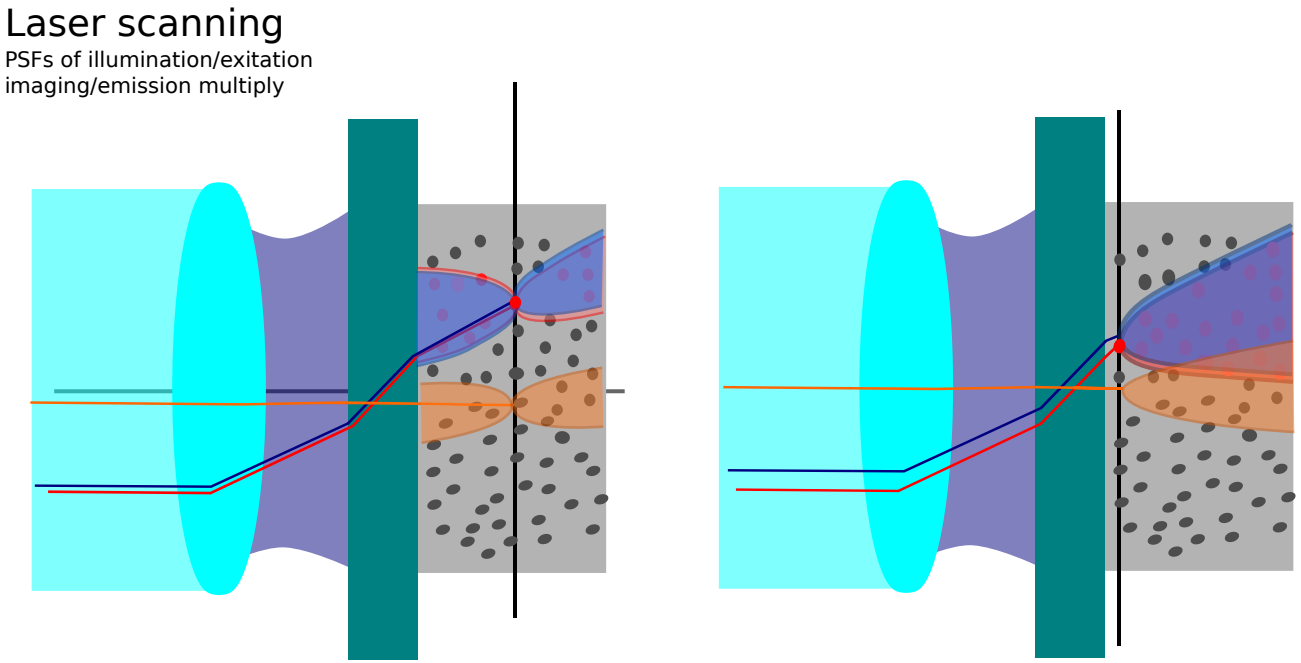
- Second tube lens: There is now a second virtual image plane, complete with magnification and everything
- Nice: Once adjusted, it follows in focus, since they use the same objective.
- Laser optics are changed, now focused to a point on the virtual image plane

Focal scanning: Moving the laser spot



- Moving the mirror moves the laser point on the virtual image, thus an illumination spot moves on the sample
- There is only a signal from that one illuminated spot: Might as well use a "detector" instead of a camera (remember a pin hole, though)
- For what follows: Points need to be scanned *and measured* separately.

Focal scanning: Effect



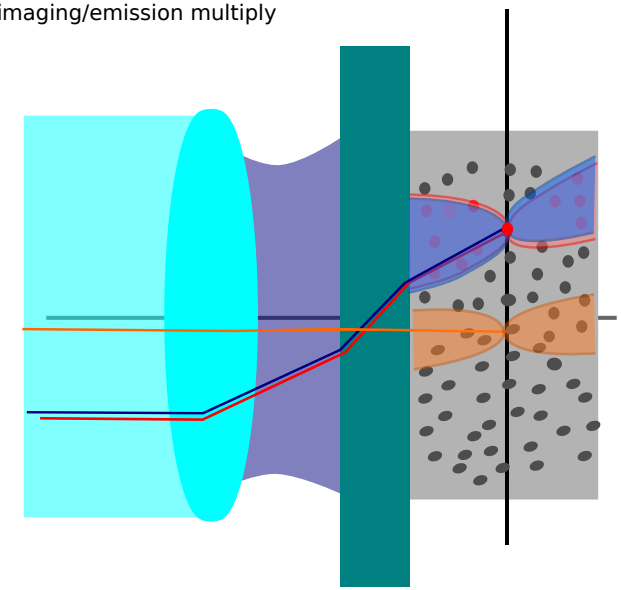
$$M(x,y) = \int_{S_z} \mathrm{PSF}_{\mathrm{em.}}(z) * (I(x,y,z) \cdot S(x,y,z))\,\mathrm{d}z \quad I(x,y,z) = I_0 \cdot \mathrm{PSF}_{\mathrm{ex.}} * \delta(x,y,z)$$

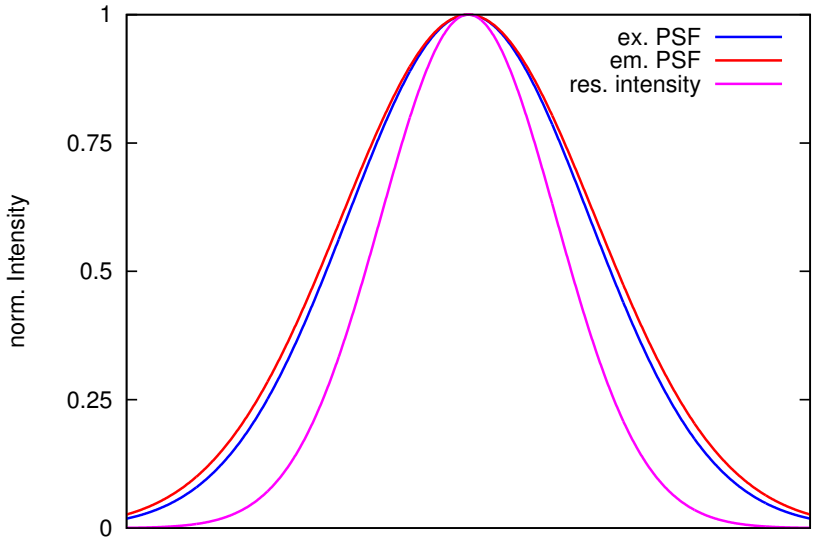
- Illumination now far from constant: Point source (delta peak) at the position the laser is focused at, folded by PSF.
- Out of focus: Not only contribute less to measurement, but also receive less light
- Sometimes relevant: PSFs differ due to wavelength

Focal scanning: Resolution improvement

Laser scanning

PSFs of illumination/exitation
imaging/emission multiply



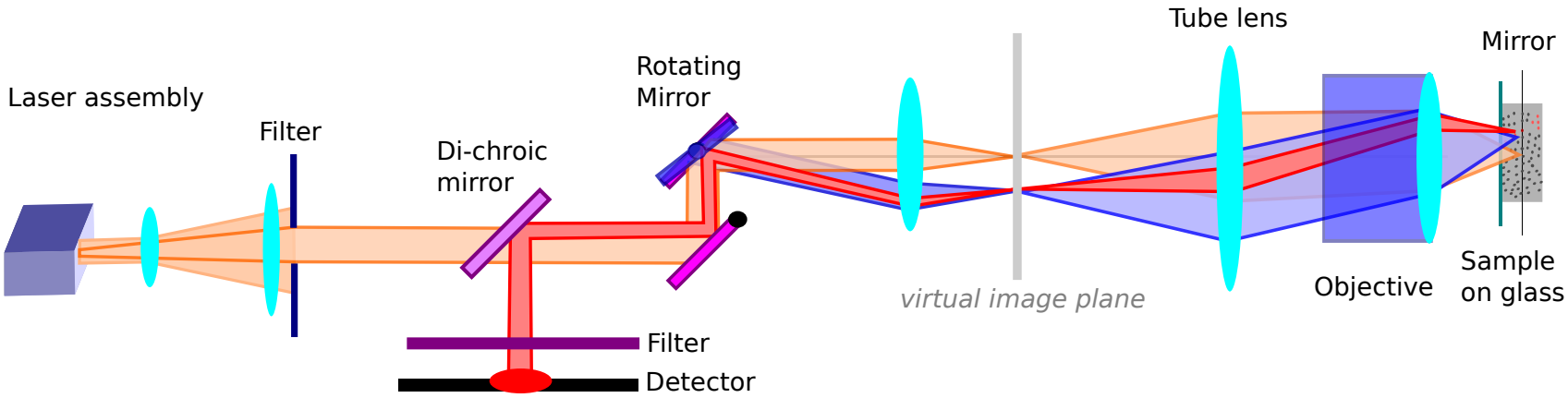


$$M(x,y) = \int_{S_z} \mathrm{PSF}_{\mathrm{em.}}(z) * (I(x,y,z) \cdot S(x,y,z))\,\mathrm{d}z \quad I(x,y,z) = I_0 \cdot \mathrm{PSF}_{\mathrm{ex.}} * \delta(x,y,z)$$

- First of all: That improvement work also lateral
- "PSF squared" means: At former FWHM, intensity M is now $\frac{1}{4}$
- New FWHM: Where ever $\sqrt{2}M_{\max}$ was in wide-field
- **For a gaussian PSF** than leads to a $\sqrt{2}$ improved resolution

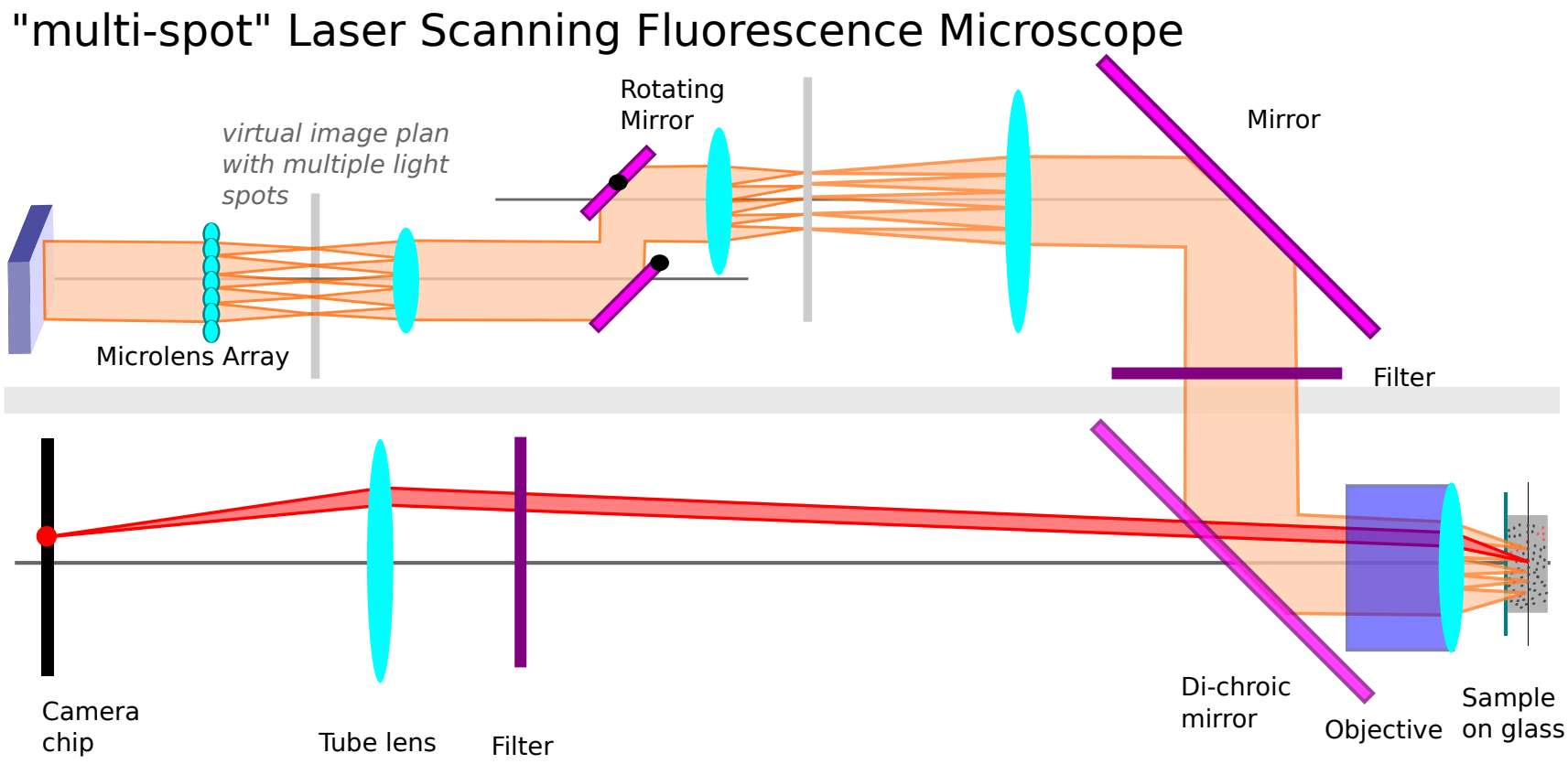
Standard one-spot confocal scanning

Laser Scanning Fluorescence Microscope
Assembly with point detector



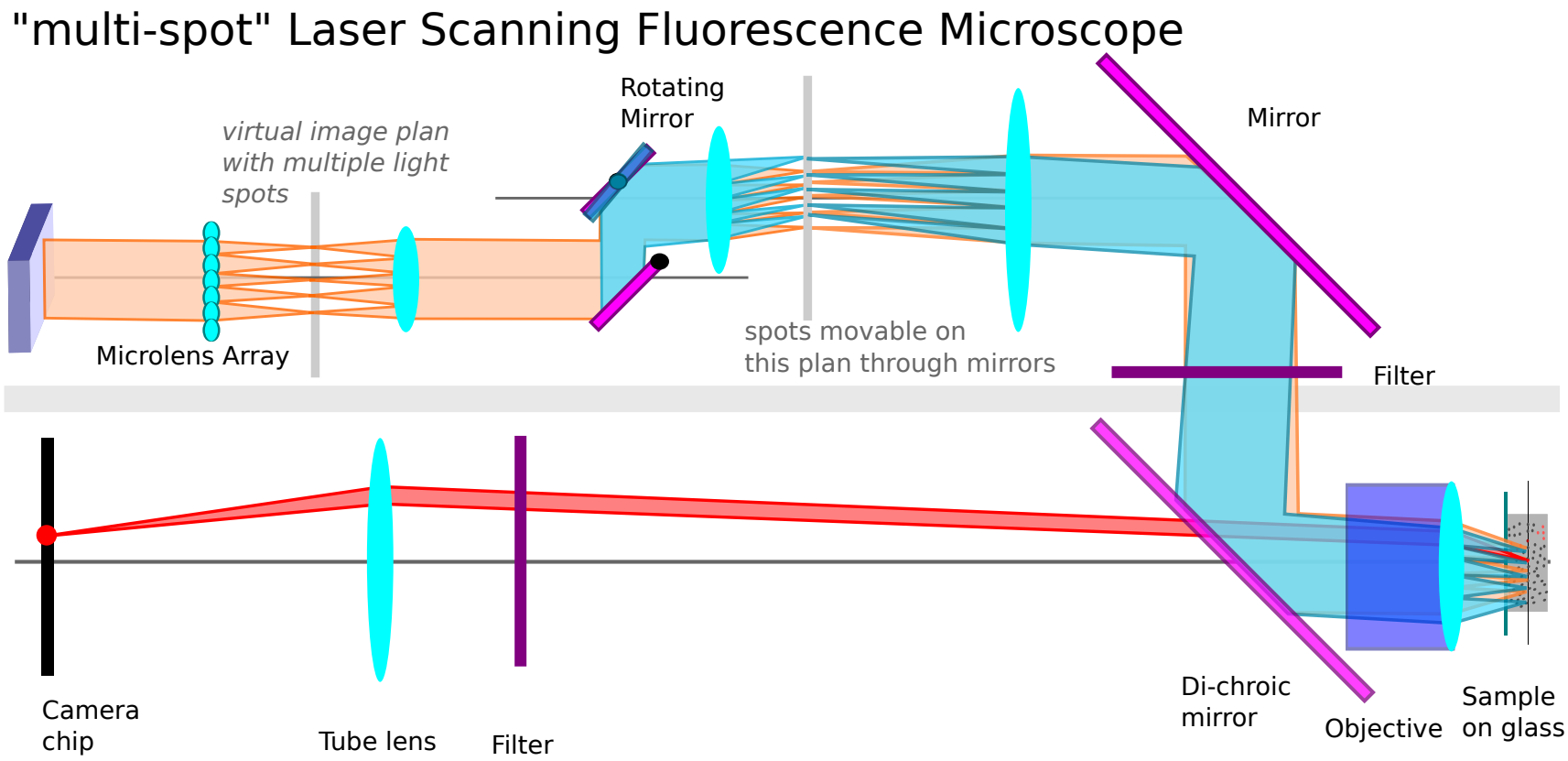
- This is a standard laser scanning microscope
- Emission and illumination now via the same mirror set
- Computer controls mirror, at each point measure the signal, maps to an image
- Detector can be much more sensitive than a camera (and e.g. measure multiple spectral lines)

Multi-spot laser-scanning



- Array of micro-lenses: Create multiple laser spots
- Mirror-assembly to move these spots

Multi-spot laser-scanning



- Multiple spots make the system faster
- Emission detection via camera and post-processing
- Often already referred to as SIM

Lecture today: "Classic" methods (TIRF, HiLo, Confocal scanning) to improve resolution via controlling how light reaches the sample.
Next lectures: Current super-resolution techniques.

Superresolution Microscopy

Techniques that allow resolution beyond the Abbe limit

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

- *Influence the illumination*: **Structured illumination microscopy (SIM)**
  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 $S(x,y,z)$.
  SIM denotes a specific technique and the general concept.
- *Use (and sometimes influence) the sample response*: **Localization Microscopy**
  Add some *property* κ to the sample, so its response to illumination can change.
  This can be switching the fluorophore (e.g. STED) or a stochastic blinking process (STROM, dSTROM).
  Localization microscopy is a somewhat vague term.
- Finally, both approaches can even be combined.