Advanced light microscopy

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1. Recapitulation: Digital images

2. Structured illumination
   - Variants of confocal microscopy
   - Background measurement by light modulation
   - SIM for lateral resolution enhancement
   - SIM microscope setup

Measurements and images

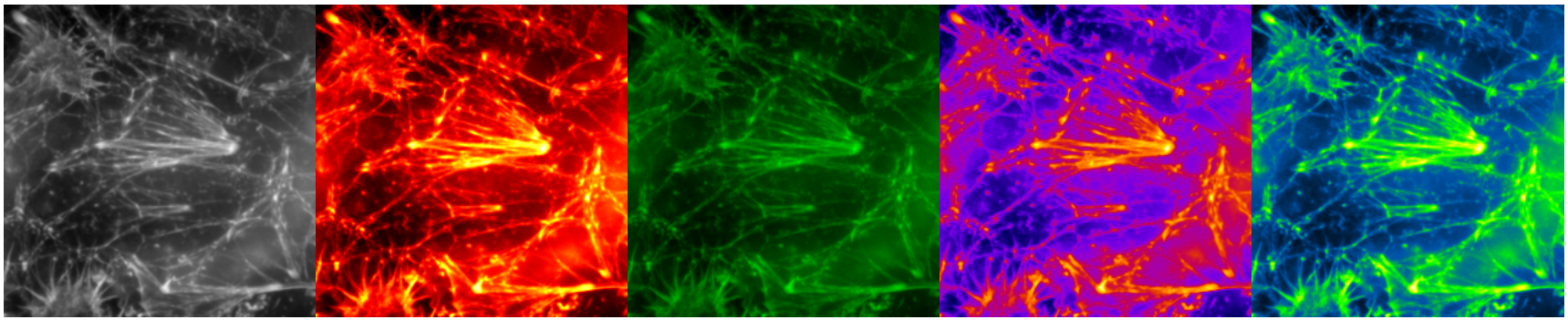
Linear encoding V_S =	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Linear intensity I =	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0

Wikipedia: Gamma Correction / Power law

12-16 bit lin. measurement → (documented/limited) processing → 8 bit bitmap
Publication as PDF, with embedded images
Systems you do not know → Display / Print-out

Linear (raw) intensity data for measurements

- Always keep and store raw data: storage is cheap
- Do not deceive: Document processing and image correction steps
- Choose a useful representation: Color-coding for different channels, for better gray-scale contrast
- Limitations of machines and humans: low contrast displays, color-blindness



Actin-labelled U2OS cell, with different lookup tables

Structured illumination microscopy

Overview: Superresolution Microscopy

Techniques that allow resolution beyond the Abbe limit

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

- **Start today**: *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.
- After the holiday break: *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.

What is SIM

SIM denotes a specific technique and the general concept.

General SIM

All methods where $I(x, y) \neq$ const., especially

- Multi-spot confocal scanning[a]
- Digital background reduction, e.g. *Zeiss Apotome*
- **Resolution:** At most confocal, i.e. $\sqrt{2}$

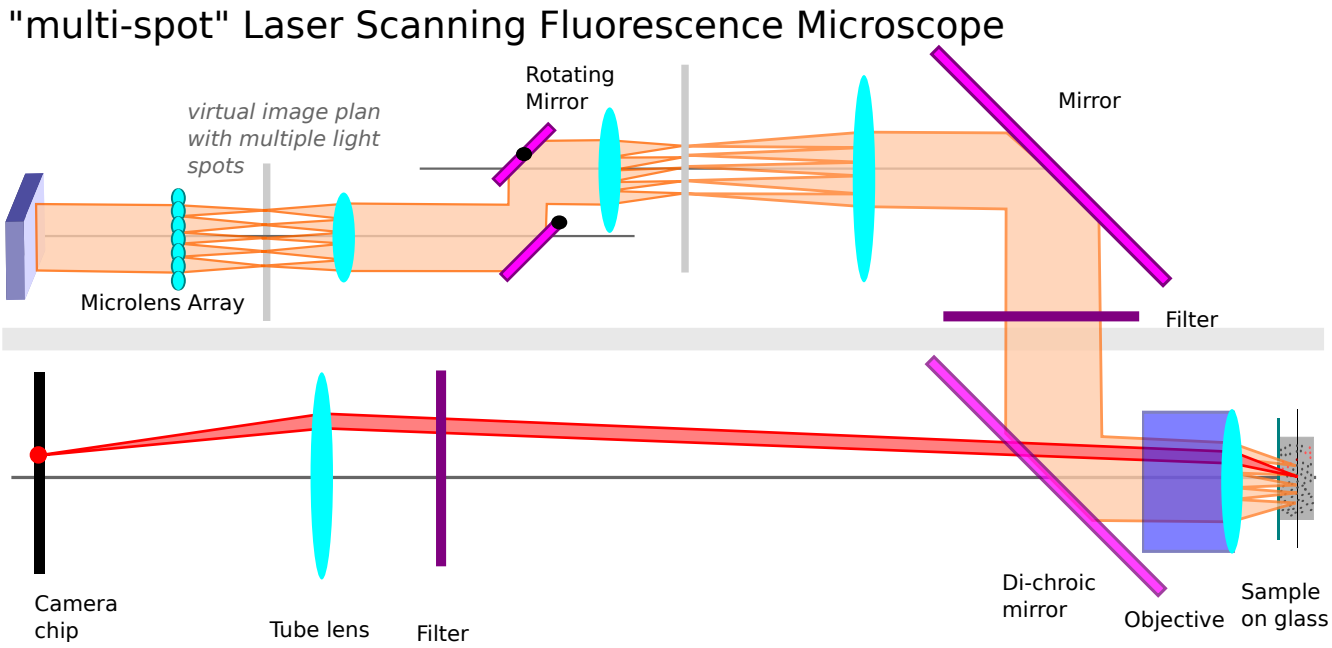
[a] Standard confocal scanning: SIM in principle, but usually not called SIM

SIM for lateral resolution enhancement

- Lateral (2D) and lateral + axial (3D) light modulation
- with multiple known illumination pattern
- Digital reconstruction of frequency components
- **Resolution:** Usually factor of 2.

Multi-spot laser-scanning

Idea: Speed up confocal scanning by using multiple spots at once.



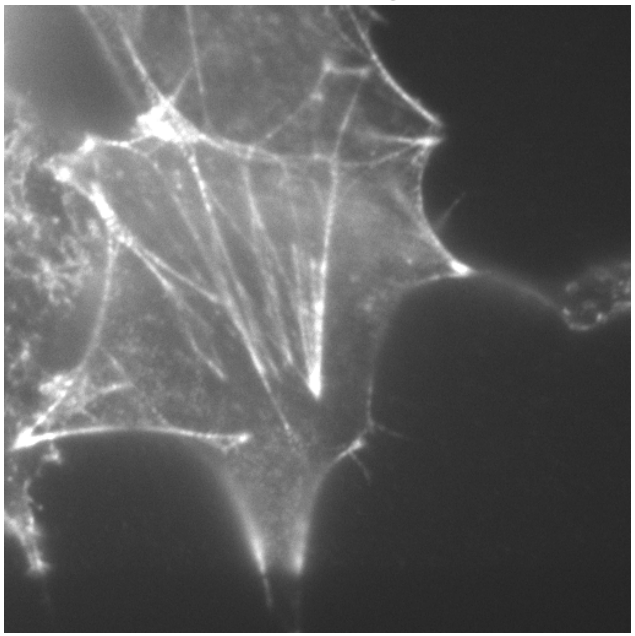
- Spot generation: Micro-lens array, SLM[1] devices
- Spot detection: Multi-PMT array (few spots), camera (more noise)
- Pinhole: digital (post-processing), second synced micro-lens array.
- System very fast, $\sqrt{2}$ confocal improvement, two-photon application.

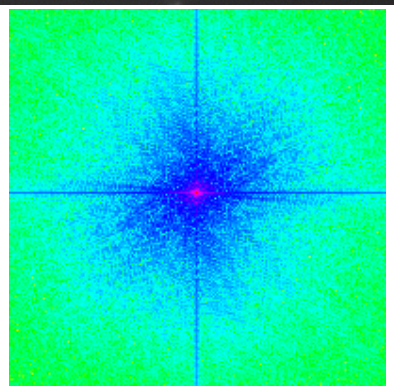
[1] Spatial light modulators

Background measurement by light modulation

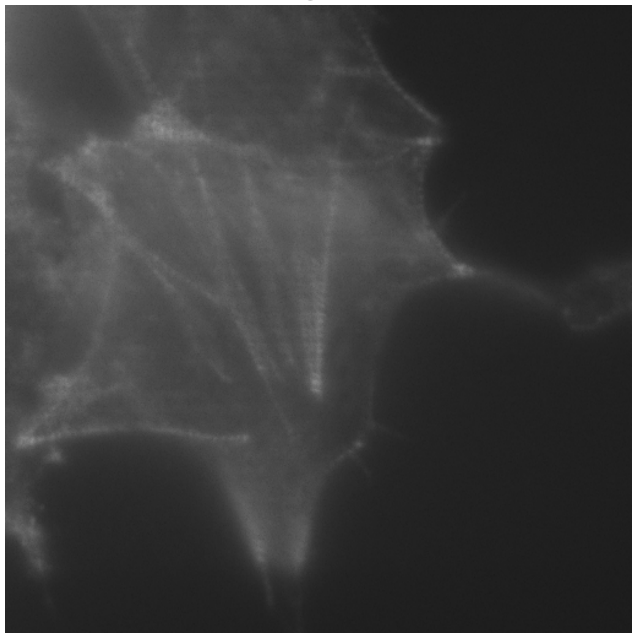
Examples for background from earlier in this lecture.
Now: How to obtain those.

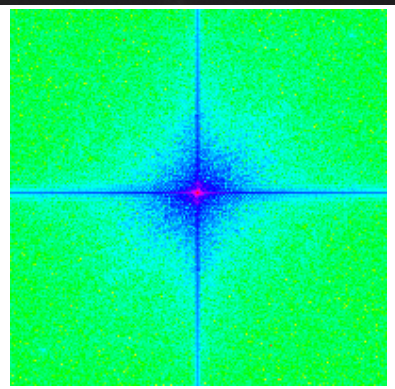
Full Image



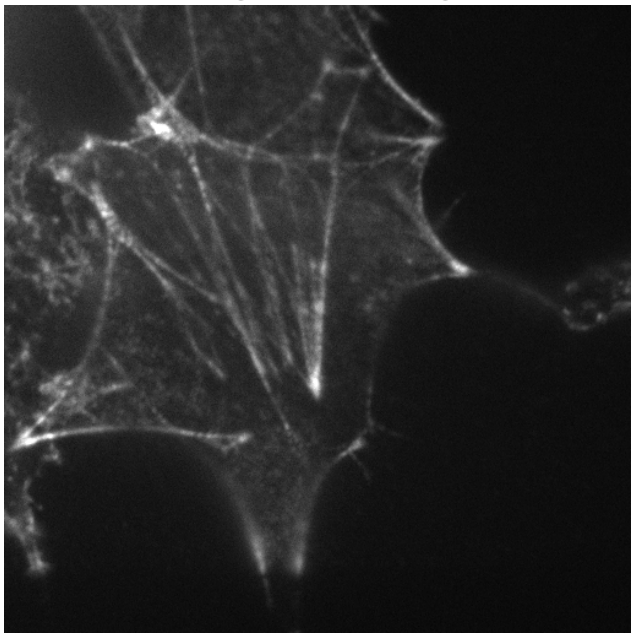


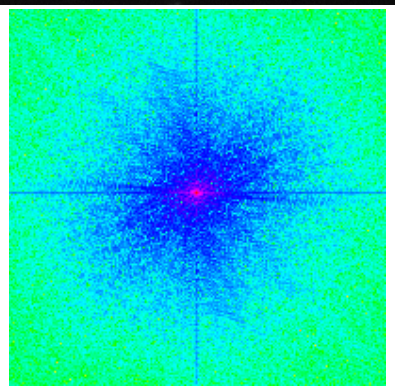
Background





Full Image - Background

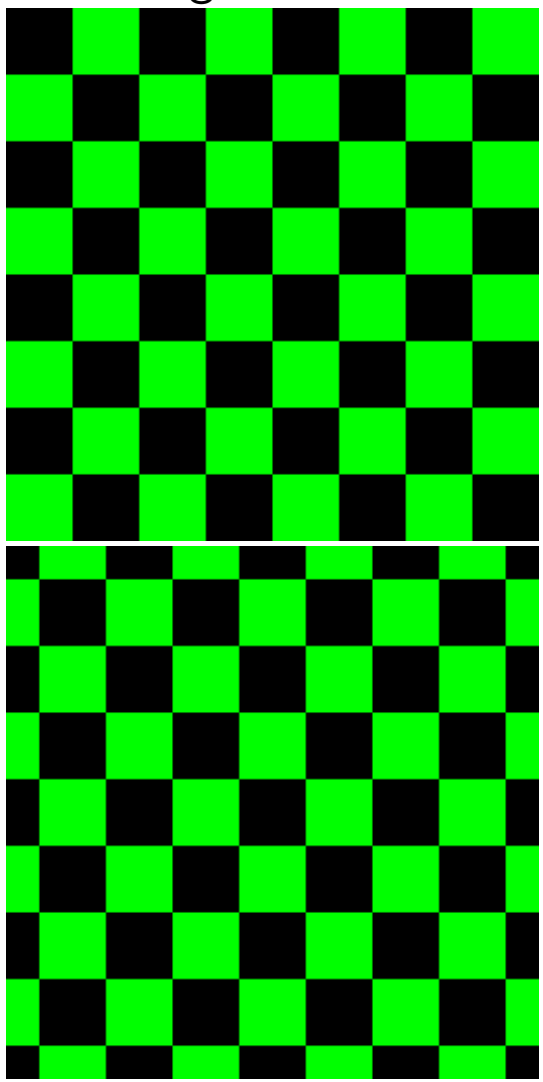




Background reduction by light modulation

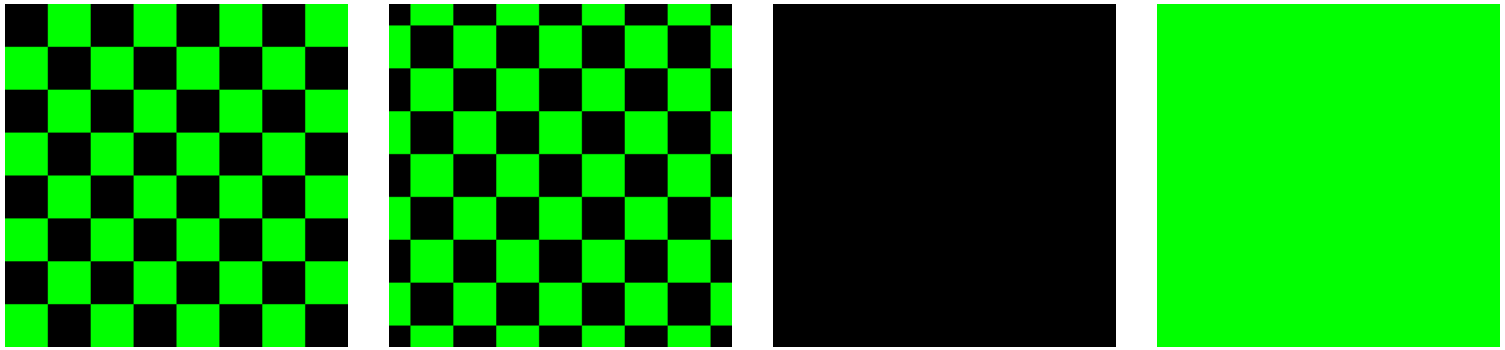
Idea: Measure background contributions, subtract them from image

- Vary $I(x, y)$ in the focal plane
- Choose e.g. a checkerboard-pattern
  $I(x, y)/I_0 =$
  $1 + \mathrm{sgn}(\sin(2\pi \cdot \kappa \cdot x + \phi_x)) \cdot \mathrm{sgn}(\sin(2\pi \cdot \kappa \cdot y + \phi_y))$
- Set the pattern spacing frequency κ close to the resolution limit
- Take multiple measurements, each time shifting the pattern by varying ϕ_x, ϕ_y.

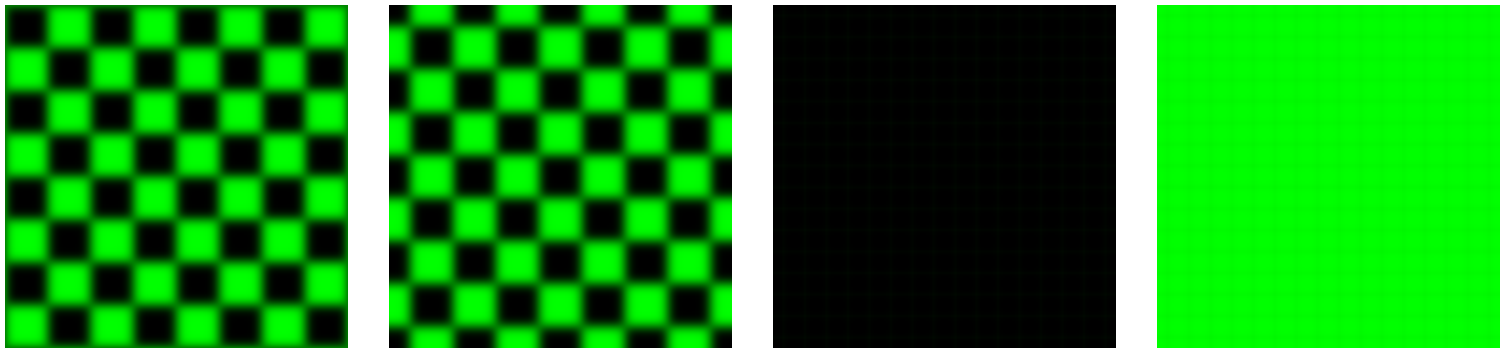


Simulation: Illumination in the focal plane and out-of-focus

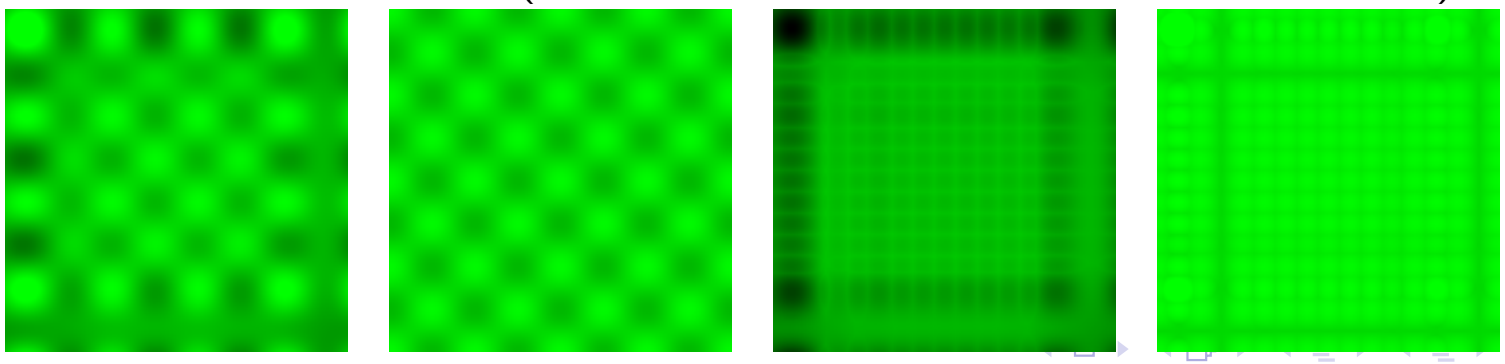
Illumination Pattern: (Pos. 1, . . . , Pos. 6, . . . , Minimum, Maximum)



Pattern in focal plane: (Pos. 1, . . . , Pos. 6, . . . , Minimum, Maximum)

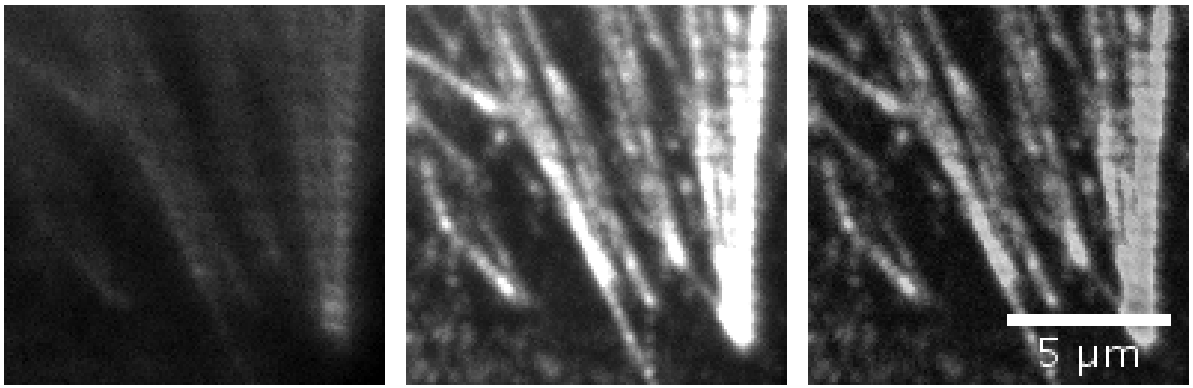


Pattern out-of-focus: (Pos. 3, . . . , Pos. 8, . . . , Minimum, Maximum)

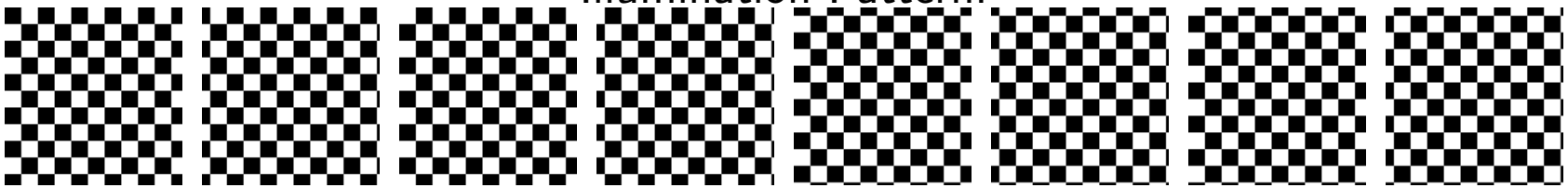


Example measurement

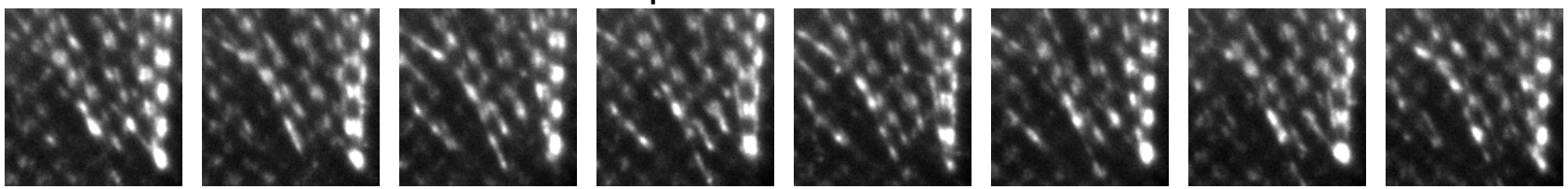
Min, Max, Result



Illumination Pattern:



Focal plane measurement:

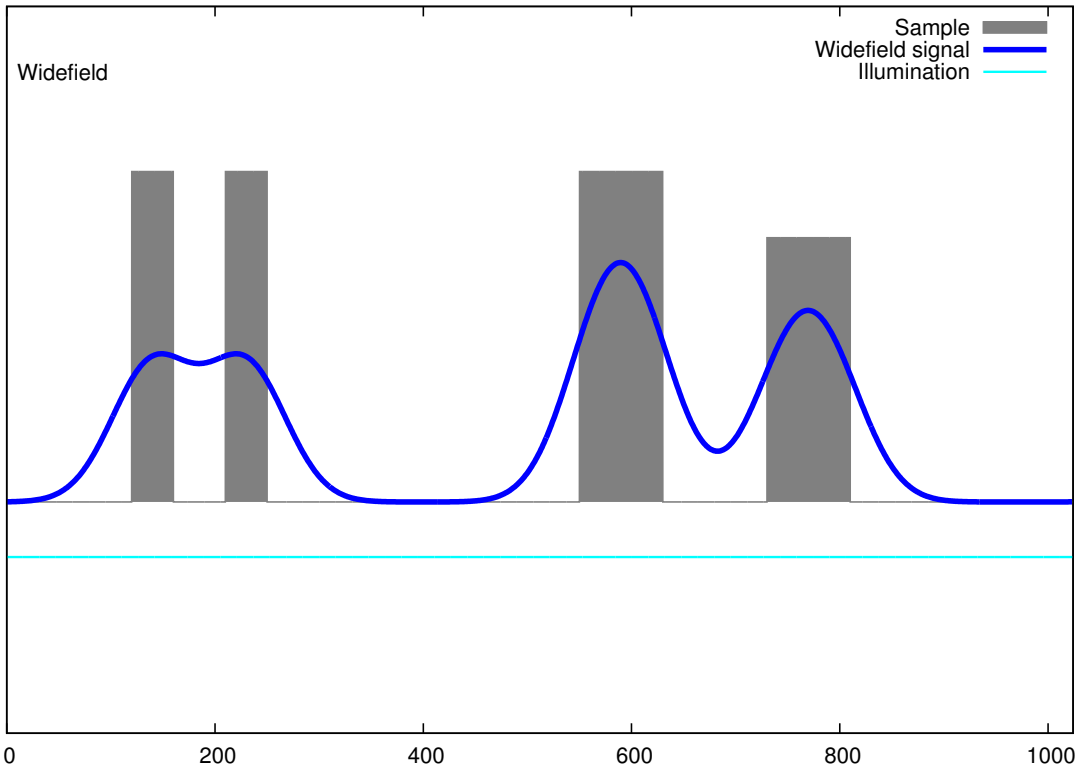


SIM for lateral resolution enhancement

Resolution enhancement through SIM

Structured illumination: Starting from wide-field...

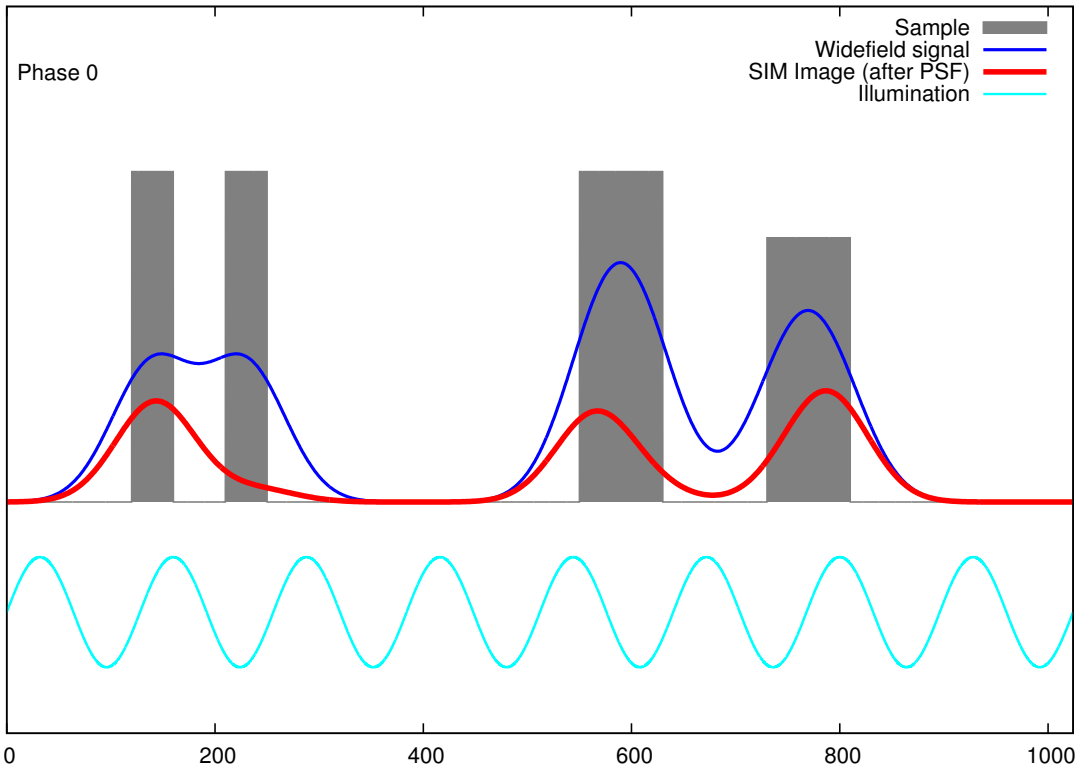
- Resolution limited by diffraction limit
- OTF cuts away high frequencies, projection to subspace
- What happens the illumination I is modulated along x, y?



Widefield image

Structured illumination: image acquisition

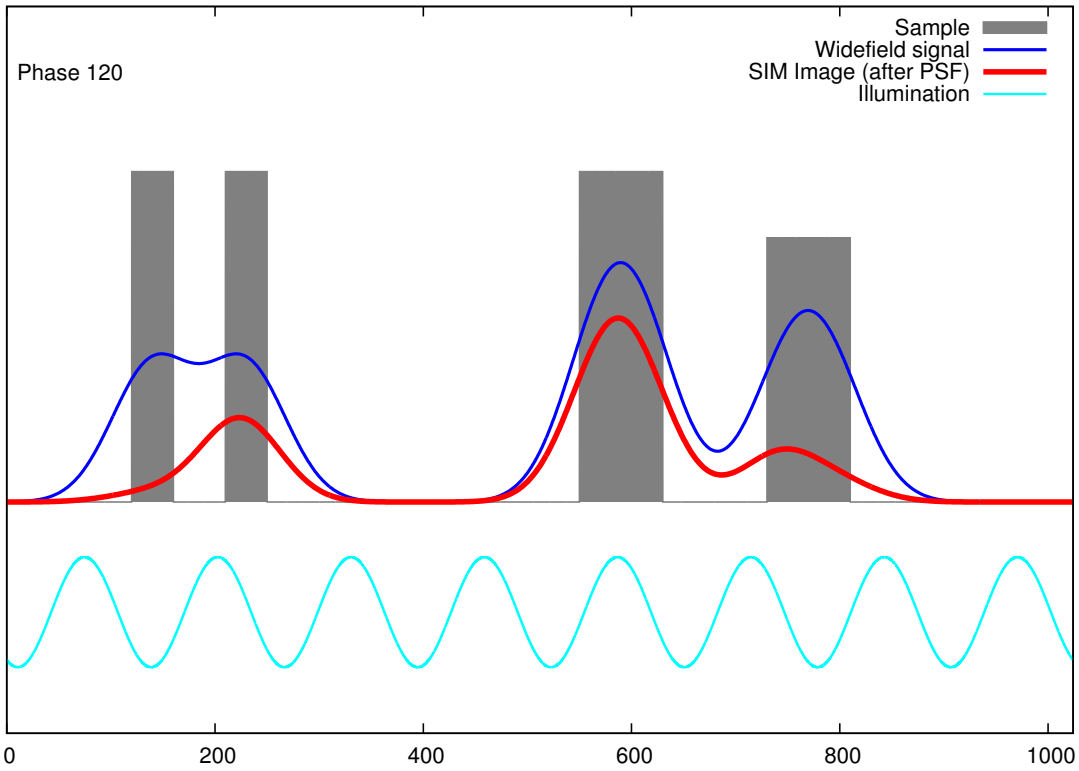
- Modulate the illuminating light with $I(x) = I_0 \cdot (1 + \sin(2\pi\kappa x + \phi))$
- Use a modulation wavelength k near the diffraction limit
- Shift the phase ϕ ...



Phase $\phi = \frac{0}{3}\pi$

Structured illumination: image acquisition

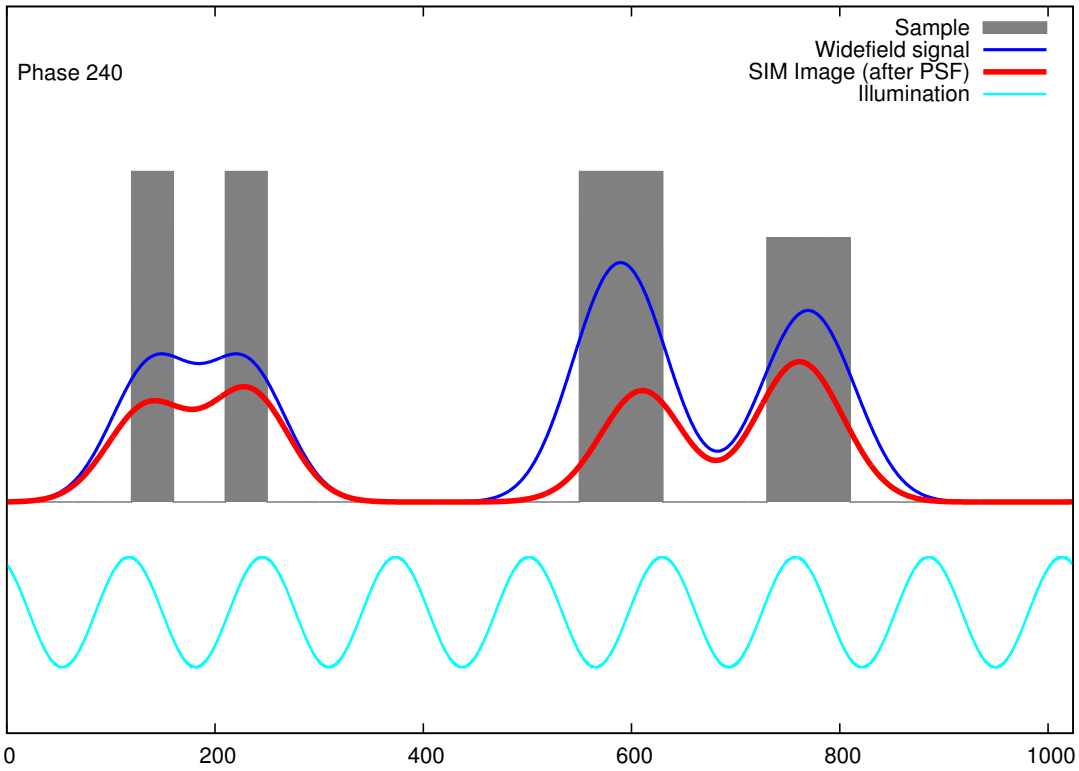
- . . . and gather additional information about the sample. . .



Phase $\phi = \frac{2}{3}\pi$

Structured illumination: image acquisition

- . . . by collecting images at three phases.
- In principle, any three phases will do (thats because any three span the same Fourier subspace).
- In reality, they should be $\frac{2}{3}\pi$ apart. (thats because measurements have errors and noise).



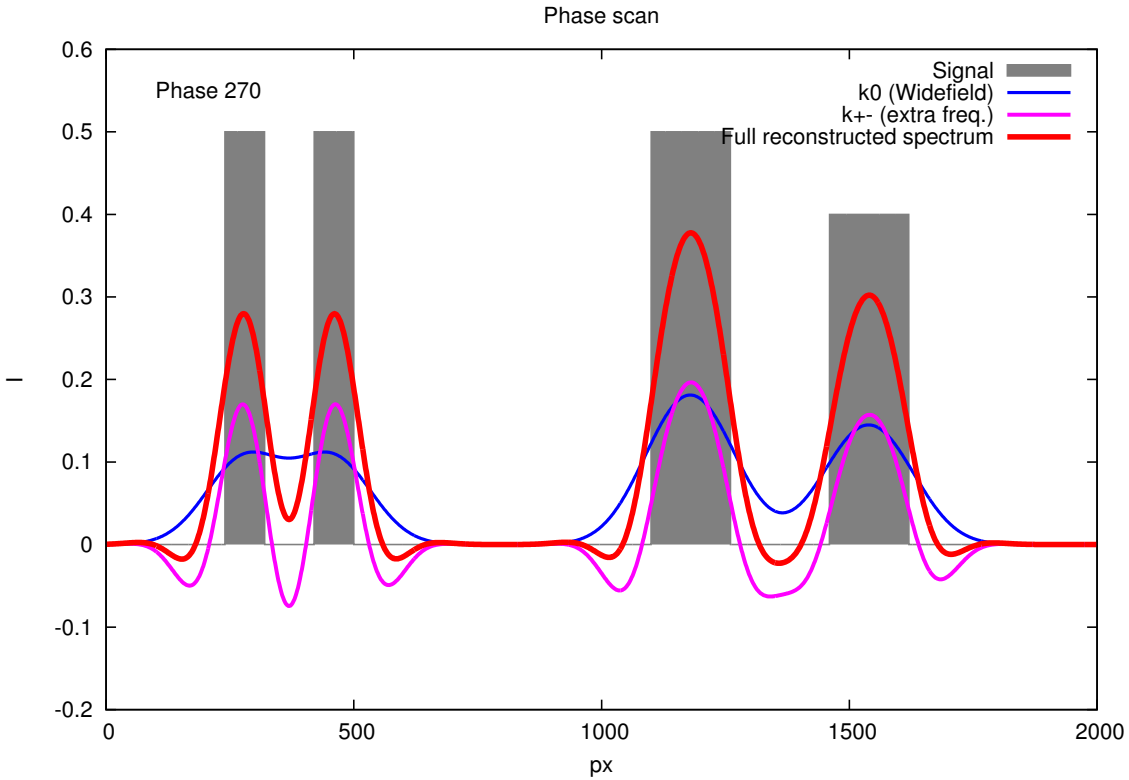
Phase: $\phi = \frac{4}{3}\pi$

Structured illumination: image reconstruction

- Recombine three images ($\phi_{0,1,2} = \frac{0}{3}\pi, \frac{2}{3}\pi, \frac{4}{3}\pi$) to one with higher resolution
- Separate contribution through M^{-1}, where

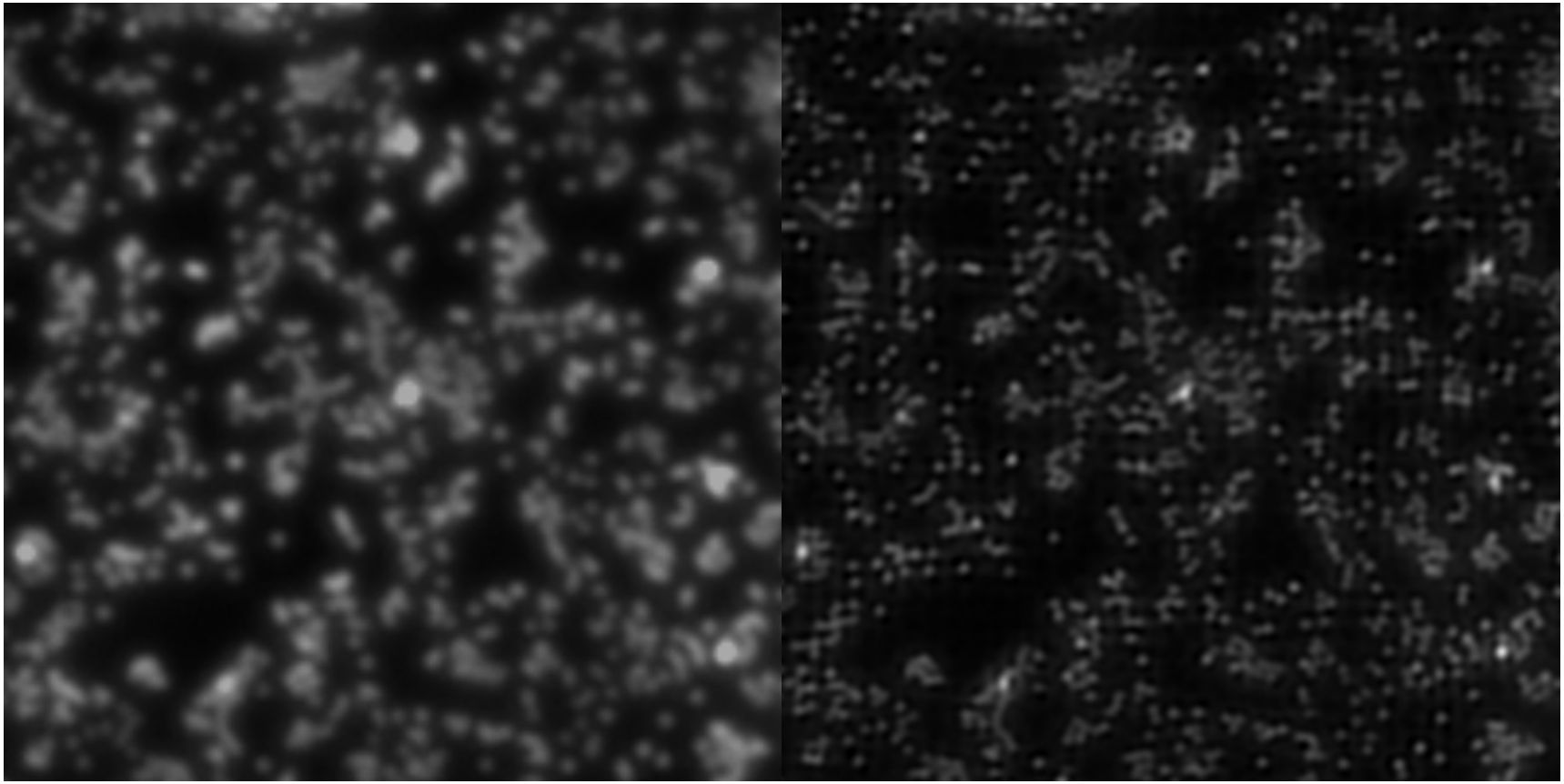
$$M = \begin{pmatrix} 1 & \frac{1}{2}e^{i\phi_0} & \frac{1}{2}e^{-i\phi_0} \\ 1 & \frac{1}{2}e^{i\phi_1} & \frac{1}{2}e^{-i\phi_1} \\ 1 & \frac{1}{2}e^{i\phi_2} & \frac{1}{2}e^{-i\phi_2} \end{pmatrix}$$

- Shift contributions to new position $\pm k_0$ in Fourier space



Reconstructed signal

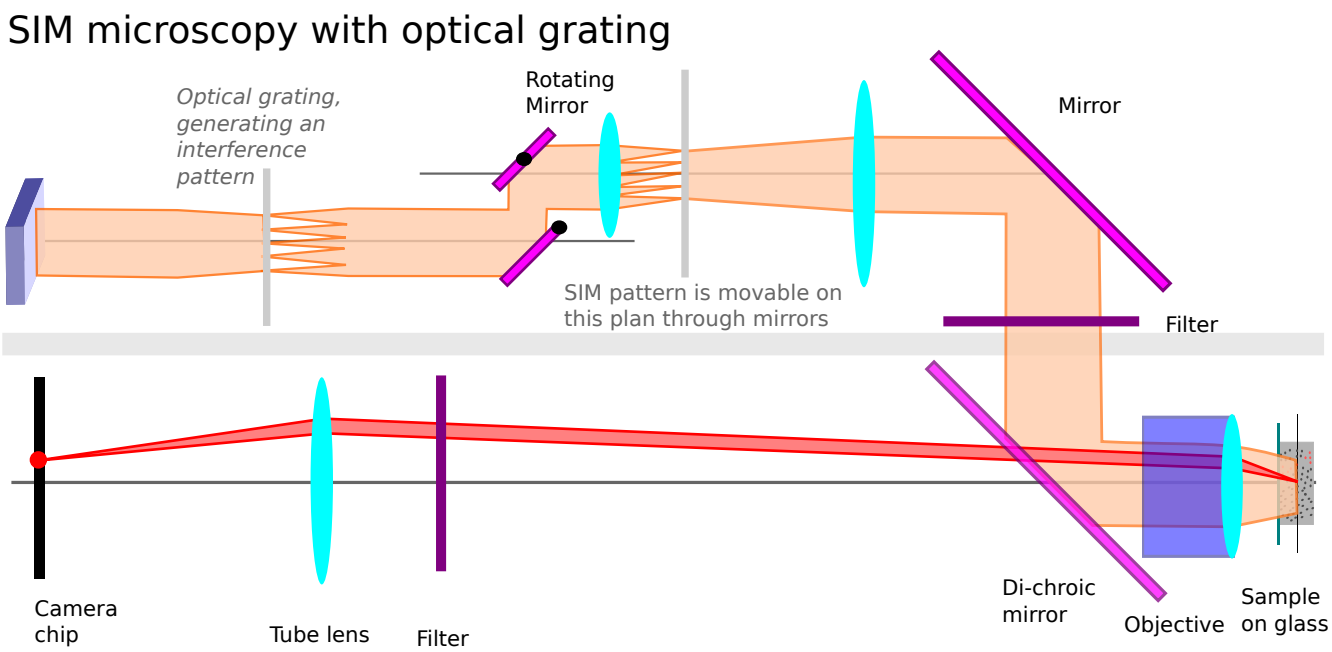
2D SIM reconstruction of test sample



- Sample: Closely-packed surface of dye-filled beads
- Widefield: Beads beyond the resolution limit
- SIM: Beads clearly visible
- Measurement on a simple 2D projection SIM
- Software written by us, open source in 1 to 2 month

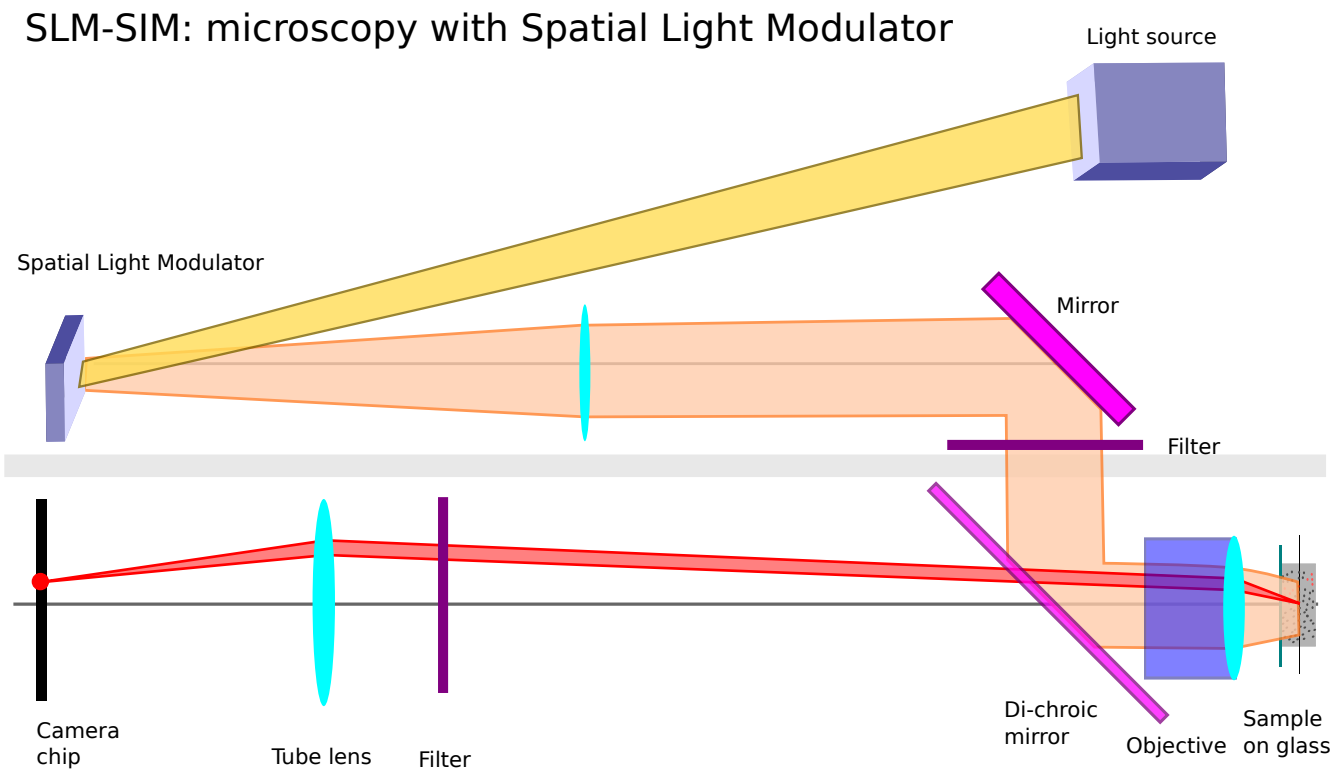
How to measure with structured illumination

SIM setup with optical grating



- First design, around 2000
- Good modulation depth
- Rather complex, mechanical (moving mirrors) setup
- Used by the machine next door

SIM setup with spatial light modulators



- SLMs widely available through projectors, etc.
- Almost free of moving components
- Very fast, low cost
- Used in low cost and/or high speed machines