Advanced light microscopy

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1. Recapitulation: SIM principle
2. SIM reconstruction algorithm
3. SIM application
4. Outlook

Structured illumination microscopy: general concept

In general, techniques where $I_l(x, y) \neq$ const. in

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

and multiple measurements M_l are combined to one image

General use of the term

Variants of multi-spot **confocal techniques** are referred to as SIM. Compared to standard confocal

- Same resolution enhancement ($\sqrt{2}$ over widefield)
- Same reduction of background
- Large speedup (depending on the specific technique used)
- Loss in sensitivity compared to PMT-based point-detectors
- Minimal (or even no) digital post-processing

Structured illumination microscopy: Lateral resolution enhancement

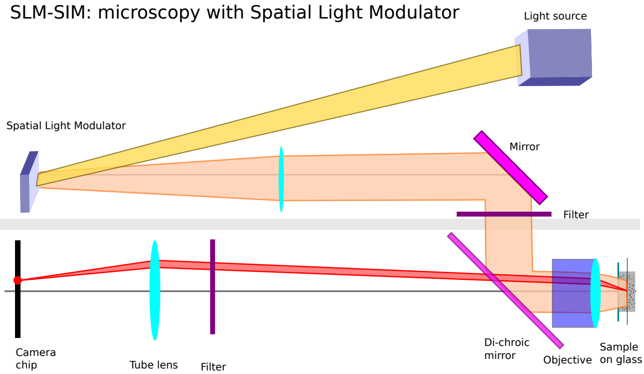
$$M_l(x,y) = \int_{S_z} \mathrm{PSF}(z) * \left(I_l(x,y,z) \cdot S(x,y,z)\right) \mathrm{d}z$$

SIM for lateral resolution enhancement

Modulation of $I_l(x, y)$ with a structure close to the resolution limit

- Realization through optical gratings or SLMs
- Multiple measurements (typ. 3 phases, 3 angles for 2D) of the same sample
- Digital reconstruction step relies on
  - Knowing the illumination patterns $I_l(x, y, z)$
  - Solving for $S(x, y)$ from eq. $M_l(x, y) = \ldots$

SIM setup with spatial light modulators



- SLMs widely available, low cost, fast
- Image acquisition standard widefield, illumination modified
- Allows to project a pattern onto the sample surface

SIM: Measurement and reconstruction

Step by step reconstruction of a SIM measurement

Step 1: Measurements

Regular illumination pattern

Use

$$I_l(x,y) = \frac{I_0}{2} \cdot [1 + \sin((2\pi \cdot p + \phi)/\kappa)]$$

where

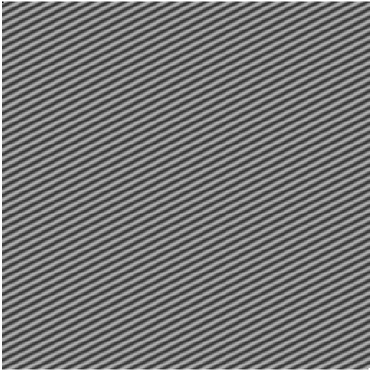
$$p = x \cdot \cos(\alpha) + y \cdot \sin(\alpha)$$

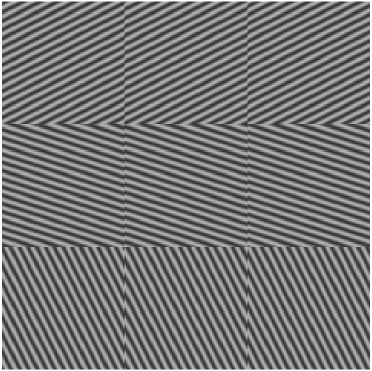
Multiple measurements

- Use multiple angles α for illumination
  Typically 3 or 4, evenly spaced
- For each angle, illuminate with (at least) 3 phases $\phi = \frac{0}{3}\pi, \frac{2}{3}\pi, \frac{4}{3}\pi$
  - Phases not evenly spaced: loss of SNR
  - More than three phases: Reconstruction gets over-defined, but still possible
- Number of angles: Full resolution enhancement along each angle α.

Step 1: Illumination pattern

Simulated illumination pattern

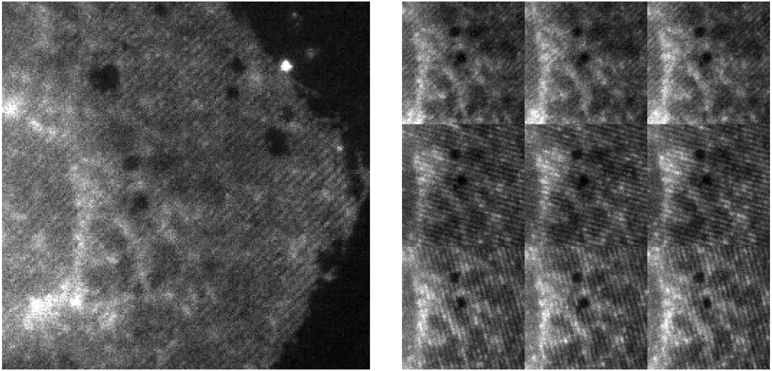




Left: First illumination pattern. Right: Montage of all illumination patterns

Step 1: Measurement (OMX next door)

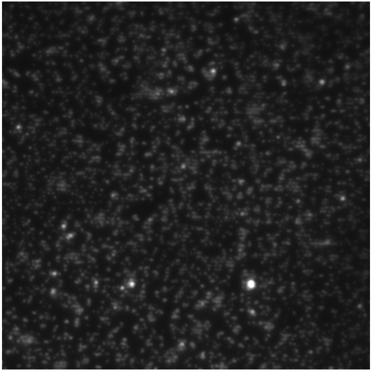
Cell measurement via the OMX. Pattern spacing: 356 nm.

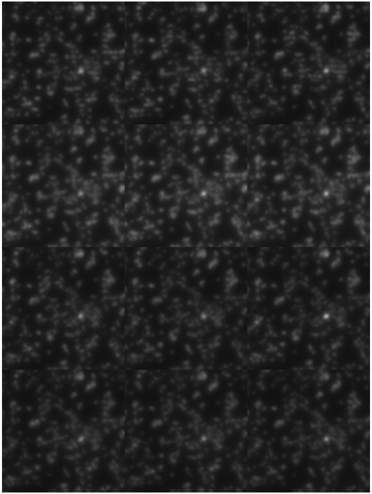


Left: First illumination pattern. Right: Cut-out, montage of all patterns

Step 1: Measurement (2D TIRF SIM)

Test surface dye-filled beads. Pattern spacing: 256 nm (at resolution limit).



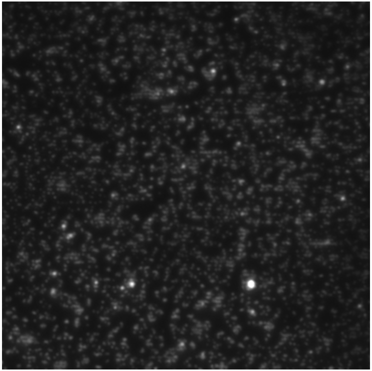


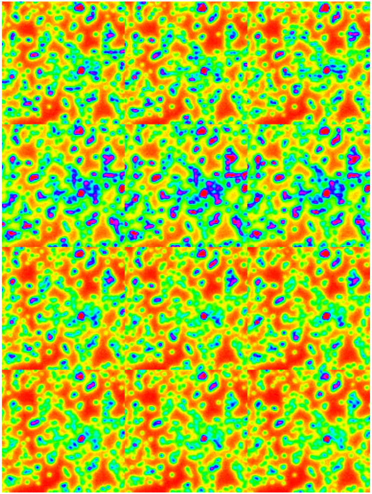
Left: First illumination pattern. Right: Cut-out, montage of all patterns

Any visible variation between the different patterns?

Step 1: Measurement (2D TIRF SIM)

Test surface dye-filled beads. Pattern spacing: 256 nm (at resolution limit).





Left: First illumination pattern. Right: Cut-out, montage with spectrum LUT

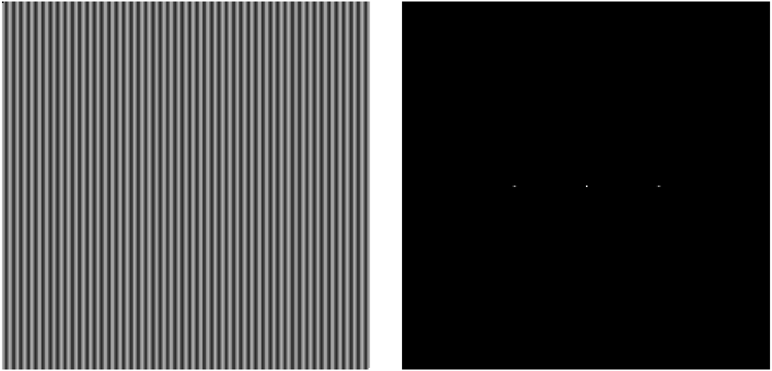
Look closely at the bright structures. . .

Step 2: Illumination in Fourier space

$$I_I(x, y) = \frac{I_0}{2} \cdot [1 + \sin((2\pi \cdot p + \phi)/\kappa)]$$

where

$$p = x \cdot \cos(\alpha) + y \cdot \sin(\alpha)$$



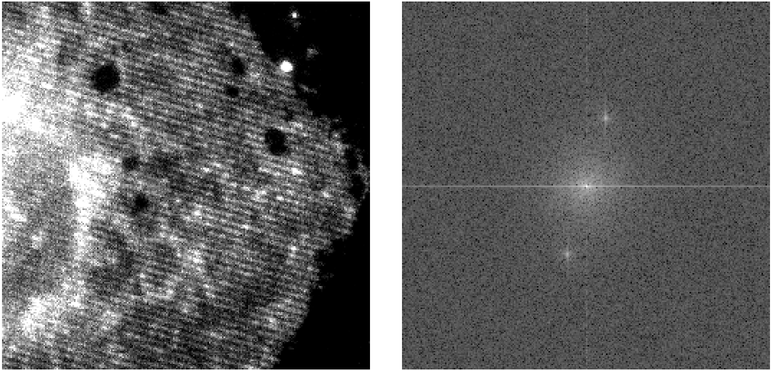
Left: Illumination pattern. Right: FFT / FHT power spectrum

Why three dots in FFT?

Step 2: Illumination in Fourier space

$$\tilde{M}_l(k_x, k_y) = \mathrm{OTF} \cdot \left(\tilde{S}(k_x, k_y) * \tilde{I}_l(k_x, k_y) \right)$$

Cell images (OMX)



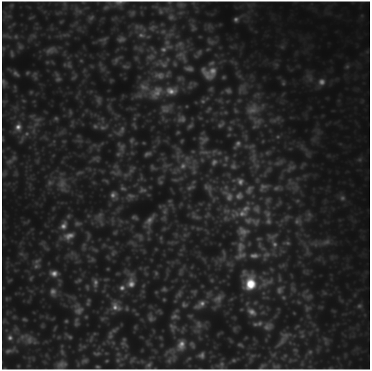
Left: Illumination pattern. Right: FFT / FHT power spectrum

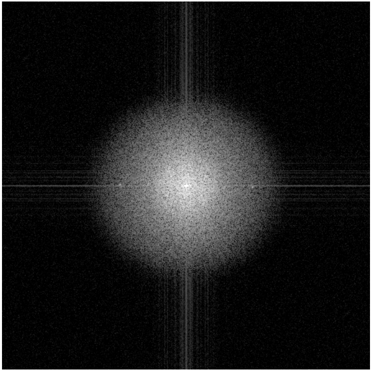
Guess how the parameters κ, α, ϕ carry over to Fourier space?

Step 2: Illumination in Fourier space

$$\tilde{M}_l(k_x, k_y) = \text{OTF} \cdot \left(\tilde{S}(k_x, k_y) * \tilde{I}_l(k_x, k_y) \right)$$

Test surface



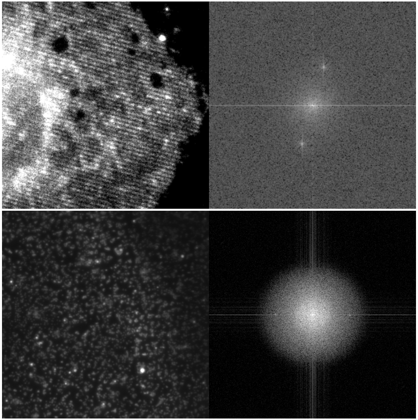


Left: Illumination pattern. Right: FFT / FHT power spectrum

Fourier space reveals the illumination pattern.

Step 2: Acquire parameters

- Three peaks in FFT power spectrum: Center "DC" and symmetric $\sin(2\pi \cdot p + \phi)$-contribution.
- Position directly translates to κ, α.
- The phase ϕ is contained in $z = r \cdot e^{i\phi}$, as the FFT yields a complex number. However, it is easily distorted by other structures in the sample.



Step 3: Decompose the measurements

Measurement

Three phases ϕ_0, ϕ_1, ϕ_2 for illumination, each row in M represents one measurement:

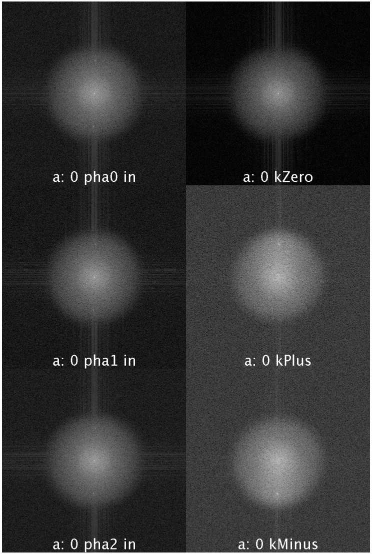
$$M = \begin{pmatrix} 1 & \frac{1}{2}e^{i\phi_0} & \frac{1}{2}e^{-i\phi_0} \\ 1 & \frac{1}{2}e^{i\phi_1} & \frac{1}{2}e^{-i\phi_1} \\ 1 & \frac{1}{2}e^{i\phi_2} & \frac{1}{2}e^{-i\phi_2} \end{pmatrix}$$

Columns: DC contributions, two symmetric contributions from the sinusoidal form.

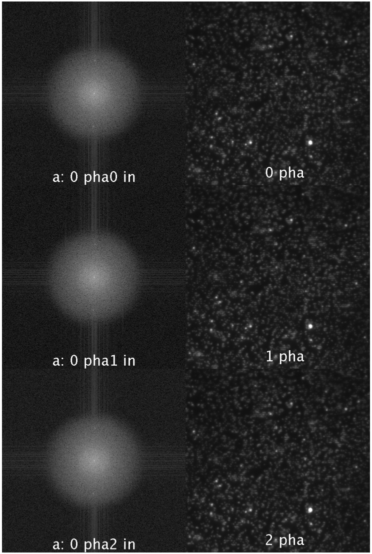
Decomposition

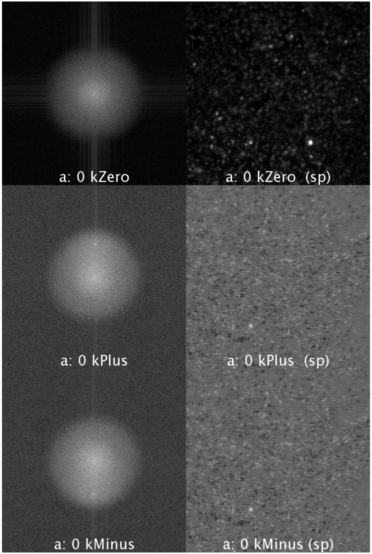
Invert[a] M to M^{-1}.
Via M^{-1}, get the DC contribution (wide-field) and higher frequencies.

[a] 3×3 matrix, otherwise Moore-Penrose pseudo inverse



Step 3: Spatial results of decomposition





DC component reconstructs to an images with wide-field characteristics.
Sinusoidal components not yet meaningful, but: κ and α have not been used so far.

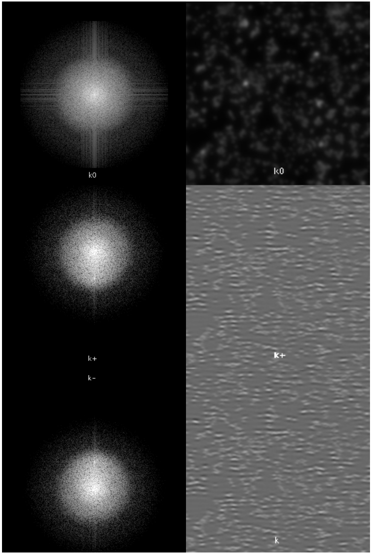
Step 4: Shifting frequency components

Compute shift

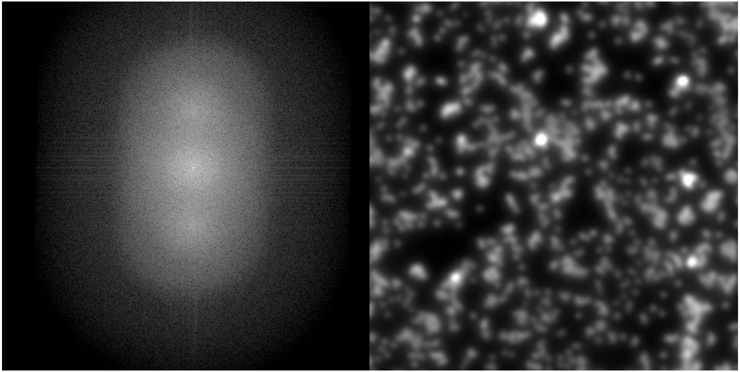
- DC component: Widefield, done.
- Two symmetric components: $\sin((2\pi \cdot p + \phi)/\kappa)$, where $p = x \cdot \cos(\alpha) + y \cdot \sin(\alpha)$
- Shift these contributions by $\pm\kappa, \alpha$

Result

- Pixels needed for shift: Resolution enhancement
- $+\kappa$ and $-\kappa$: contain the same information by definition
- Spatial transformation: Structure becomes apparent



Step 3: Added frequency components



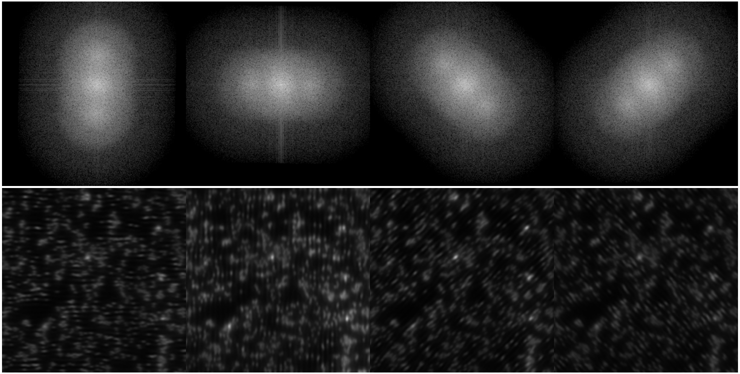
Add up the DC and shifted sinusoidal components. Result: Resolution-enhancement along illumination pattern direction α.

Interim result

- Sinusoidal pattern[1], 3 contributions in Fourier space
- Matrix inversion, decomposition and shift
- Resolution enhancement along the pattern direction
- **Number of phases:** At least 3, more lead to an over-defined matrix
- When measuring these 3 phases, κ and α have to be fixed, phases should be evenly spaced.
- **Number of angles:** Arbitrary, but usually 3 or 4 to fill the Fourier space.
- κ may change between angles α.
- **Resolution enhancement:** Directly given by pattern spacing κ.

[1] Other pattern: How does their Fourier transform look like?

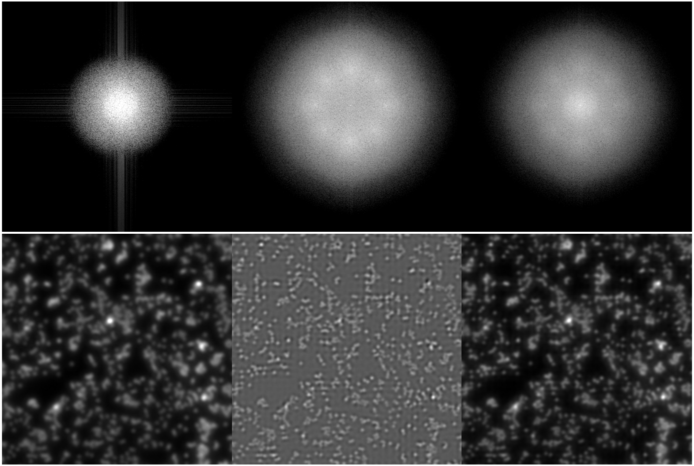
Step 4: Measure for multiple angles α



Three angles α. Resolution enhancement along each angle of the pattern. Next step: Combine these spectra.

Step 4: Combine all angles

$$\tilde{M}_l(k_x, k_y) = \mathrm{OTF}(k_x, k_y) \cdot \left(\tilde{S}_l(k_x, k_y) * \tilde{I}(k_x, k_y)\right)$$



Low, high and all frequency components

Low frequency widefield, high frequency additional information, all frequencies combined. Close, but not quite right. What is missing?

Step 5: OTF frequency filtering

$$\tilde{M}_l(k_x, k_y) = \mathrm{OTF} \cdot \left(\tilde{S}(k_x, k_y) * \tilde{I}_l(k_x, k_y)\right)$$

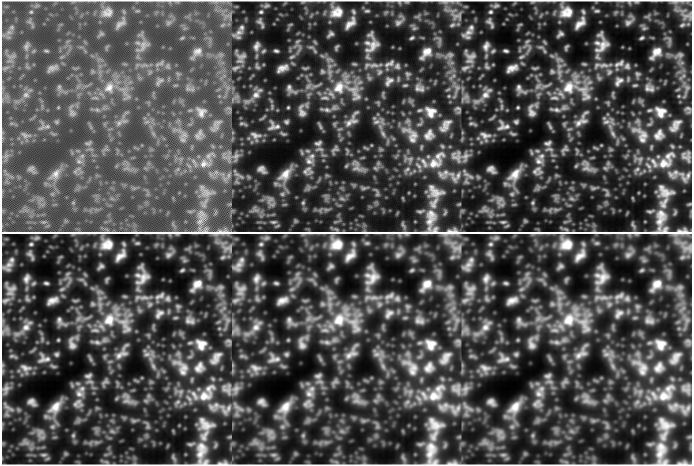
- So far, result looks better, but not quite right
- Transfer function: Not only limits resolution, but dampens high frequencies
- This leads to problems when shifting components around
- Solution: Divide by OTF in frequency domain.
- However: Low to zero regions in OTF will cause artifacts
- Full solution: frequency filtering

Step 5: (modified/generalized) Wiener filter

$$\tilde{R}(k_x, k_y) = \sum_l \frac{OTF(k_x, k_y)\tilde{M}_l(k_x, k_y)}{\mathrm{OTF}^2(k_x, k_y) + \omega}$$

- Multiply by OTF and divide by OTF^2, thus: Result without frequency dampening
- Numerator: One contribution of OTF is multiplied in post-processing, one is inherent to the measurement.
- Parameter ω: Artificial high-frequency dampening.
  Dominates in regions of a low OTF, with quadratic response.
- Determining the OTF: Big difference between 2D and 3D:
  2D: Use any OTF (Gaussian, Bessel, ...) with a somewhat matching FWHM
  3D: Measure a quite exact OTF along the axial direction.
- There are more involved filtering methods available (e.g. iterative ones like total variations)
  But: *No further information is generated by filtering.*

Step 5: Result of Wiener filtering



Reconstruction with different Wiener filter settings:

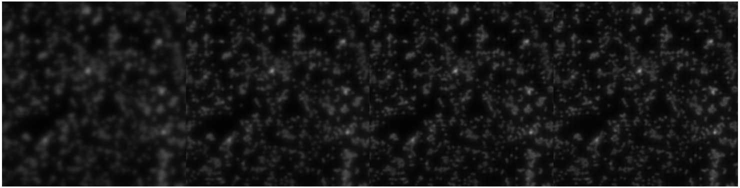
0.05, 0.1, 0.5, 2, 10, 50

Step 5: Apotization

$$\tilde{S}'(k_x, k_y) = \mathrm{APO} \cdot \sum_l \frac{OTF(k_x, k_y)\tilde{M}_l(k_x, k_y)}{\mathrm{OTF}^2(k_x, k_y) + \omega}$$

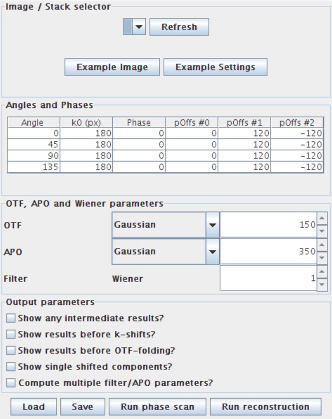
- After the filtering step, $R(k_x, k_y)$ now has no "natural" dampening of higher frequencies.
- This leads to harsh contrasts that would not be obtained by a higher resolution microscope
- Fix: Multiply an apotization function APO to the result
- APO: An artificial OTF, dampening high frequency components the same way a microscope would
- FWHM of the APO: motivated by the higher resolution limit set through the original OTF, κ and (to a lesser degree) ω.
- In practice: Start with $\mathrm{FWHM}_{\mathrm{APO}} = 2 \cdot \mathrm{FWHM}_{\mathrm{OTF}}$, tweak ω and APO with lots of leeway.

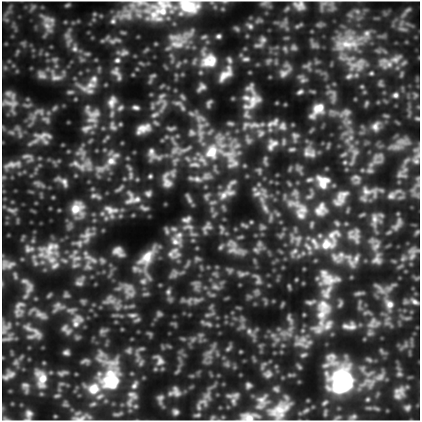
Step 5: Result of Wiener filtering



Reconstruction with different apotization settings: 50, 100, 200, 400

Step 5: Final result





SIM reconstruction software and final result

Algorithm presented today

- Sinusoidal form needed
- 2D since ≈ 2000,
  3D since ≈ 2008.
- In Bielefeld: OMX and self-made setup
- In development: Free, open-source reconstruction software

Blind SIM

- Idea: Set $\sum_{l=1}^{N} I_l = \text{const.}$
- This leaves
  $M_l(x, y) = \text{PSF} * (I_l(x, y) \cdot S(x, y))$
  with
  $I_N = 1 - \sum_{l=1}^{N-1} I_l$
- That is solvable for $I_1, \ldots, I_{N-1}, S$
- Non-linear system
- Solvable: e.g. non-linear variants of conjugate gradient

Outlook

- This concludes structured illumination
- Wolfgang: Demonstration of the OMX in SIM-mode
- (maybe): Demonstration of the SLM-SIM setup
- Next lectures: Localization-based methods:
  STED, STORM, PALM,...