Nanoscopy

Marcel, Wolfgang

Biomolecular Photonics, Bielefeld University

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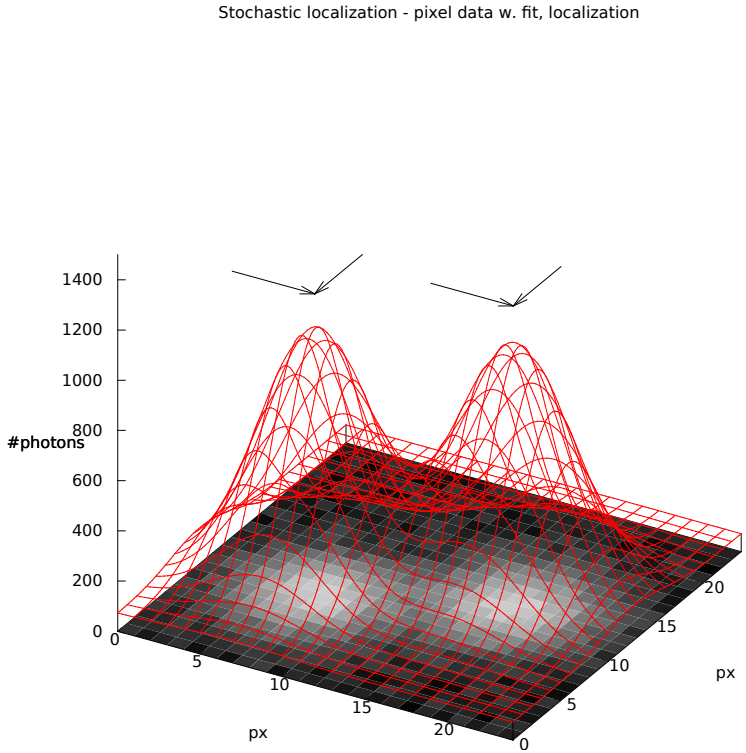
1. Visualization: Stochastic localization microscopy
   - Position fitting
   - Rendering: Histogram
   - Rendering: Gaussian
2. Sample preparation
   - dSTORM vs. PALM
3. Resolution
   - Localization precision
   - Nyquist criterion
   - Axial localization
4. Conclusion of lecture series

Fitting the distribution

For each frame:

- Fit peak to possible emitters, store value and fit precision for
  - position
  - intensity
  - FWHM
  - frame number
- List of detected fluorophores *is* the result
- Image: Visualization of that list



Rendering localization positions

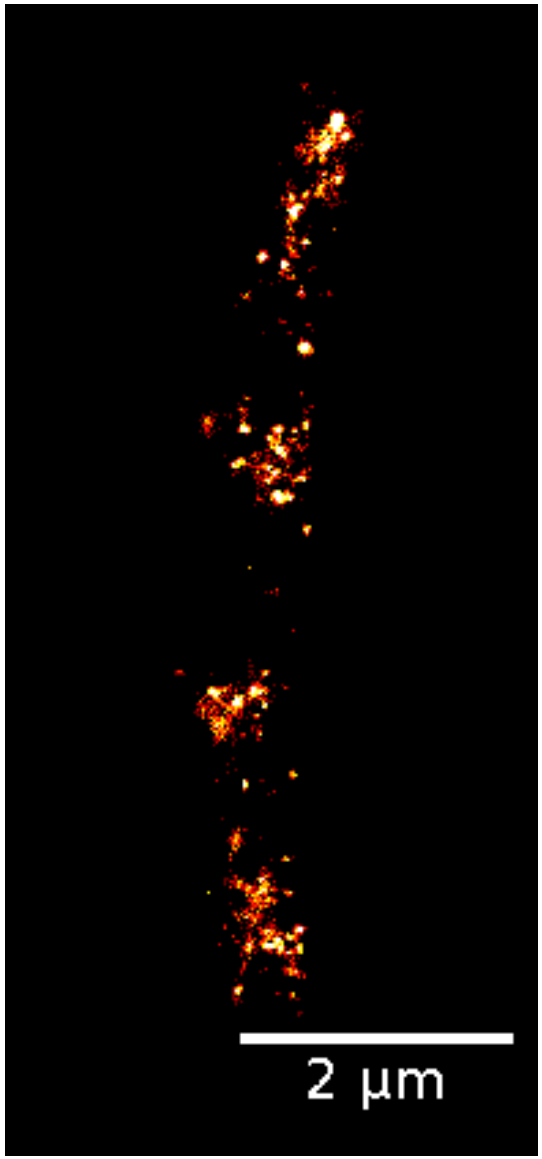
Visualization:

- Turn the list of localizations into an image
- How to represent each fluorophore?
- How to set pixel / image size
- How to threat intensities
- Use the fit precision estimates?

```
6966.77 10.1132 5923.34 17.315 -151.784 0 9688.75 5241 188.585
6778.68 6.47998 7475.78 23.0865 -562.665 1 36278.6 9259.17 245.981
6725.97 10.3507 10405.5 8.34478 54.3637 2 17511.4 17177 351.025
6782.05 6.07382 7559.35 20.4446 -487.329 2 32082.6 8142.57 249.057
6989.41 12.6998 5924.84 9.89775 62.7747 2 12546.4 7381.59 277.083
6804.42 7.45535 7512.92 8.87406 -54.9022 3 20344.3 9970.51 282.386
6714.75 15.2313 10416.8 7.38348 211.07 3 17162.4 14801.6 332.954
6955.22 10.4373 5914.71 8.47817 53.5744 3 16324.8 9339.59 285.982
6731.95 12.5114 10405.9 7.14781 159.513 4 18730.1 14455.1 336.5
6763.59 7.18383 7435.3 26.342 -545.649 4 28854.3 6171.68 244.153
6941.63 9.13922 5910.92 10.3466 -38.7977 5 15571.6 11329.1 298.601
11765.1 29.5416 8492.25 13.6638 205.952 5 5907.87 3047.12 189.595
6980.2 9.45848 5899.71 7.91641 46.1368 6 18411.1 8668.03 281.287
6712.6 10.2943 10445.3 9.67783 11.7355 6 14928.9 10899.7 301.305
6725.83 8.56592 10441.7 9.75265 -40.6563 7 17303.6 10535.1 313.114
6775.45 7.26754 7444.94 26.7514 -592.896 7 32818.2 6298.01 229.06
6741.04 7.06112 10536.2 26.247 -526.379 8 28750.2 11477 287.245
6750.37 7.21097 7440.37 26.2891 -641.478 8 39254.6 7320.34 220.456
6460.63 7.23665 9253.83 6.121 45.1064 9 28971.4 14640.1 338.809
6930.49 10.3356 5930.72 12.4692 -54.3073 9 12405.8 9498.85 290.937
6585.77 6.13533 10232.7 21.0343 -787.719 10 92143.8 40221.2 277.436
6781.9 6.92959 7511.48 25.4188 -542.699 10 30762.5 12189.9 264.652
```

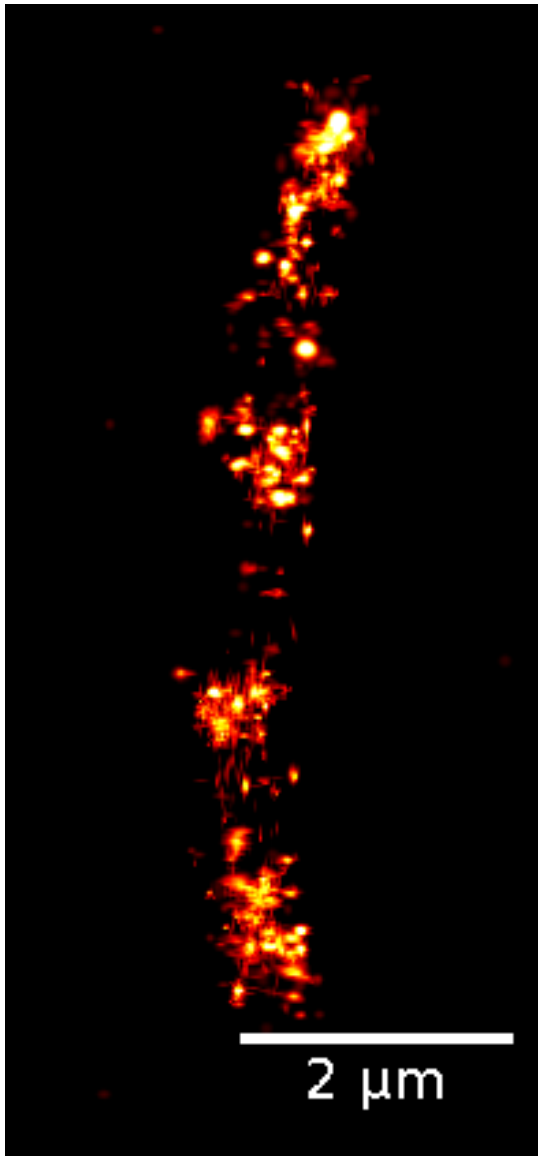
Rendering: Localization histogram

- Choose a pixel size (e.g 20nm)
- Create an empty / black image
- For each localization, find its corresponding pixel
- Increase that pixel by $+1$
- Result: 2D histogram of localizations
- Visually pleasing: Smooth with filtering
- What about precision?



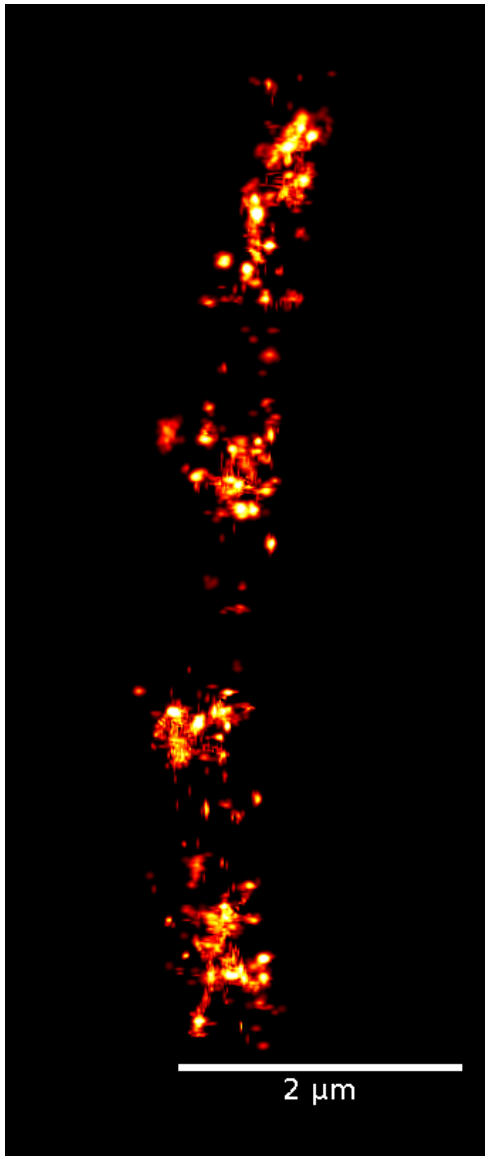
Rendering: Gauss corresponding to localization fit

- Choose a pixel size (here 20nm)
- Create an empty / black image
- For each localization, draw a Gaussian distribution
- Position given by localization
- FWHM given by fit precision
- Intensity: From fit or fixed distribution



Rendering: Gauss corresponding to localization fit

- Rendering becomes independent of pixel size (for small enough pixels)
- Smooth(er) image: Choose smaller pixels (here: 10nm)
- Localization precision automatically included
- No post-rendering filters (Gaussian, etc.) needed



Sample preparation: How to measure single fluorophores

Blinking fluorophores

Only a fraction of all fluorophores can emit in each frame

dSTORM
direct stochastic optical reconstruction microscopy

- One wavelength excitation
- Drive fluorophores into triplet-state
- Match time spend in triplet to acquisition
- Second wavelength: Stimulate triplet to ground state transition

PALM
Photo-activated localization microscopy

- Two wavelengths: excitation + switching
- Use fluorescent proteins which first have to be switched on
- Switching is stochastic: Only some proteins activate
- Activated proteins bleach, next set is activated

Post-processing does not differ (same software for dSTORM, PALM)

Resolution

How to define resolution

Localization precision

Fit precision depends on photon count

$$r \sim \frac{\sigma_{\text{PSF}}}{\sqrt{N_{\text{photon}}}}$$

Localization precision

$$\sigma_{\text{loc}} = \sqrt{\frac{\sigma_{\text{PSF}}^2}{N} + \frac{a^2/12}{N} + \frac{8\pi\sigma_{\text{PSF}}^2 b^2}{a^2 N}}$$

where:

a : Pixel size

b : Background noise

N : N_{Photon}

Fundamental limits:

- $\sqrt{N}$ from Photon quantization / shot noise: Precision of histogram, thus fit
- FWHM of PSF: Distribution width of histogram, thus also of fit

Technical considerations

- Quantum efficiency: emitted vs. counted photons
- Camera readout noise

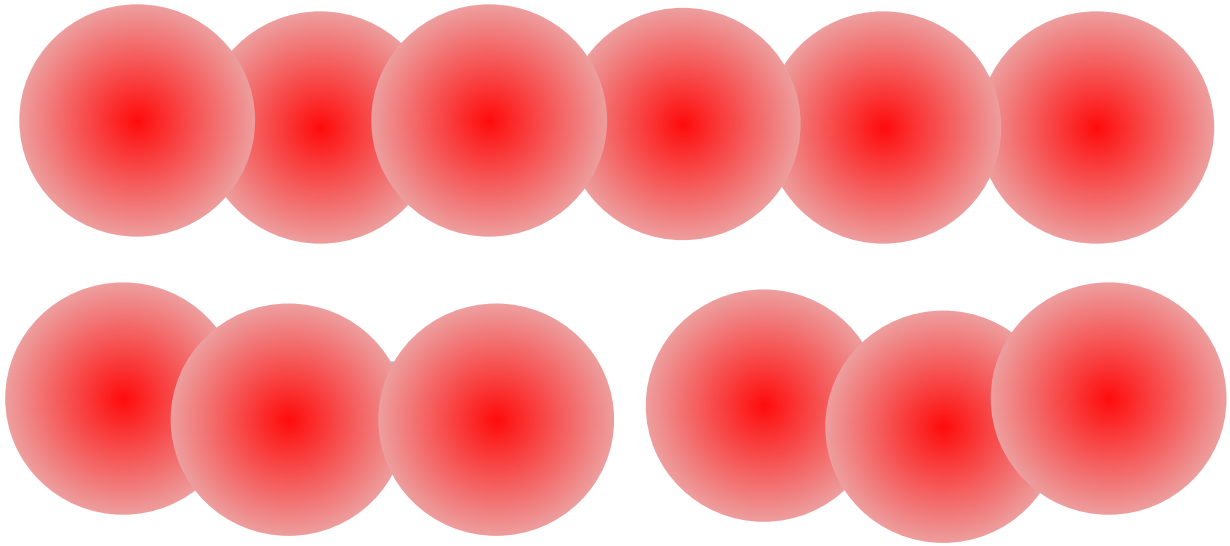
Sampling: Nyquist criterion

Labeling density:

- Resolution is *not* only given by localization precision
- Also important: Labeling density
- On the right: Structure to resolved...

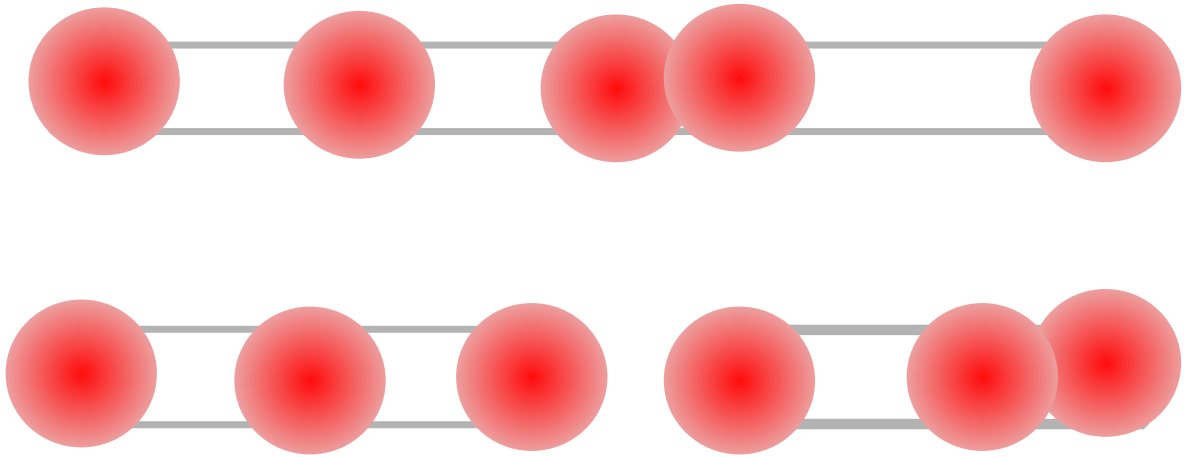
Sampling: Nyquist criterion

- Structure resolved by (rather imprecise) localizations
- Make localization more precise...



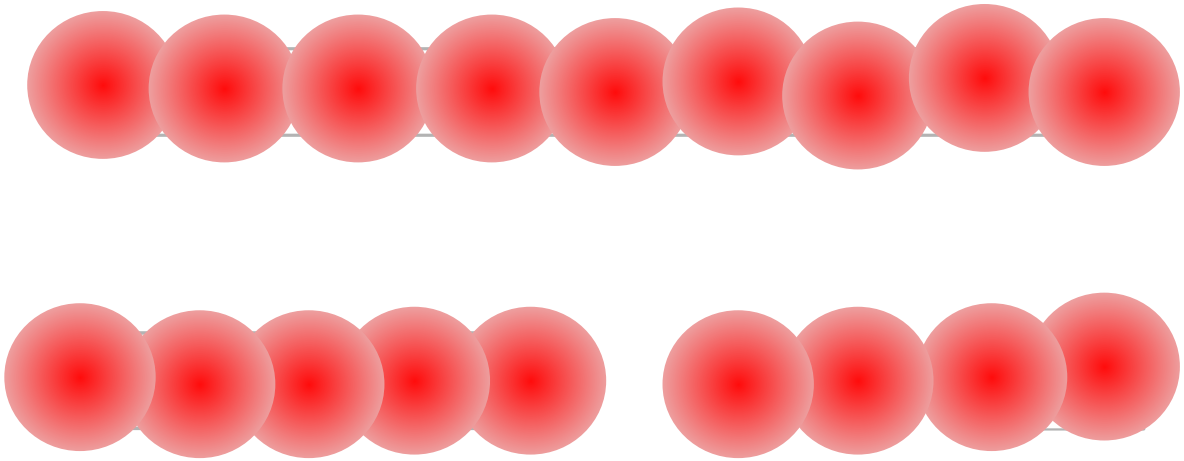
Sampling: Nyquist criterion

- Now: precise localization
- But: not enough fluorophores
- Thus: Structure not visible



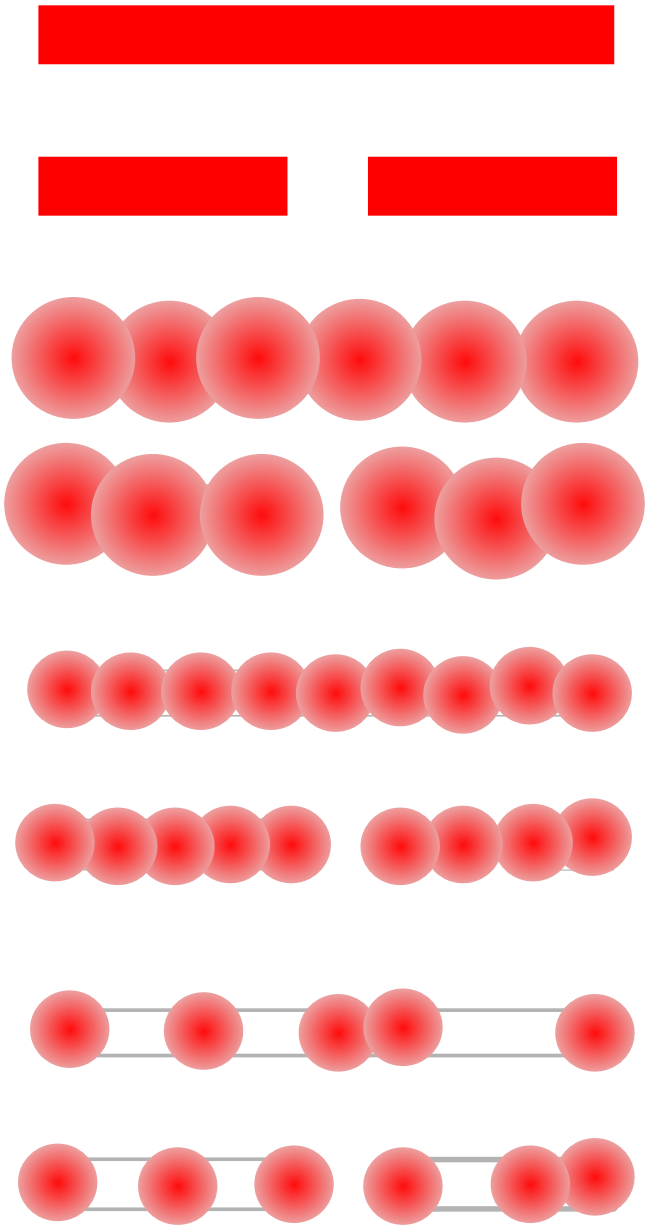
Sampling: Nyquist criterion

- Finally: Enough localization to clearly show the structure



Sampling: Nyquist criterion

- Nyquist criterion: Average distance of fluorophores at most half of the rendered size.
- Important: Trade-off rendering precision / labeling density
- Tweak: Labeling density / single emitter fitting



Extension to 3D

Axial localization

- Out of focus localization: Wide PSF
- Only interested in one plane: Reject fits over threshold width
- Axial position: Introduce aspherical element
  cylidrical lens, optical grating, SLM, . . .
- Out-of-focus PSF not symmetric
  Calibrate to z-offset, map asymmetry to z-position

Conclusion

What wasn't covered

- Non-fluorescent signals: Raman, CARS, SERS, ...
- Alternative labels: Quantum dots, SERS particles, ...
- Other stochastic reconstruction approaches: SOFI, 3b, ...
- More complex microscope layouts: 4π, multi-objective
- Image analysis: Co-localization, ...
- Non-imaging techniques: FCS, ...
- Biological and non-biological application