Nanoscopy

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SoSe 2015

1. Localization microscopy
   - Working principle
   - Variants
2. Deterministic localization microscopy
   - STED principle
   - STED PSF
3. Stochastic localization microscopy
   - Position fitting
   - Algorithm

Working principle

$$M_I(x,y) = \int_{S_z} \mathrm{PSF}(x,y,z) * (I(x,y,z) \cdot S_I(x,y,z))\,\mathrm{d}z \tag{1}$$

- Use multiple measurements M_I of the same fluorophores.
- Illumination $I(x,y,z)$ fixed: confocal or wide-field
- Use a change in the fluorophore $S_I(x,y,z)$ itself (i.e. toggle the fluorescence)
- Introducing **non-linearity** is important: allows for higher resolution improvement
- Two quite distinct approaches: Deterministic and stochastic

Localization Microscopy

Deterministic methods

- Actively switch fluorophores into an on- or off-state
- Resolution improvement: switching occurs non-linear
- Typically uses (modified) confocal illumination
- Signal processing quite similar to standard confocal

Stochastic methods

- Highly sensitive wide-field detection, high frame rate
- Induce fluorophore blinking: Most fluorophores off in most frames
- Ensure that the PSFs of remaining events to not overlap
- Post-process by detecting positions

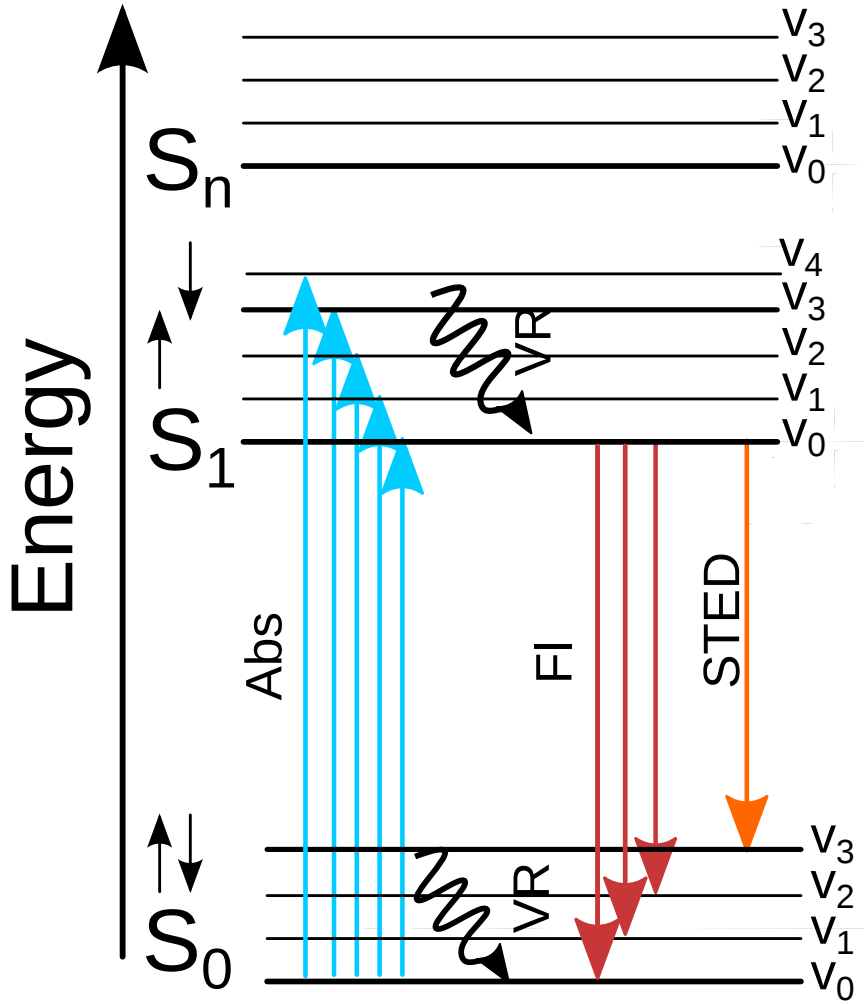
Nobel prize 2014 for both STED (determ.) and PALM (stoch.)

Deterministic RESOLFT-techniques, here: STED

STED

Three wavelength:

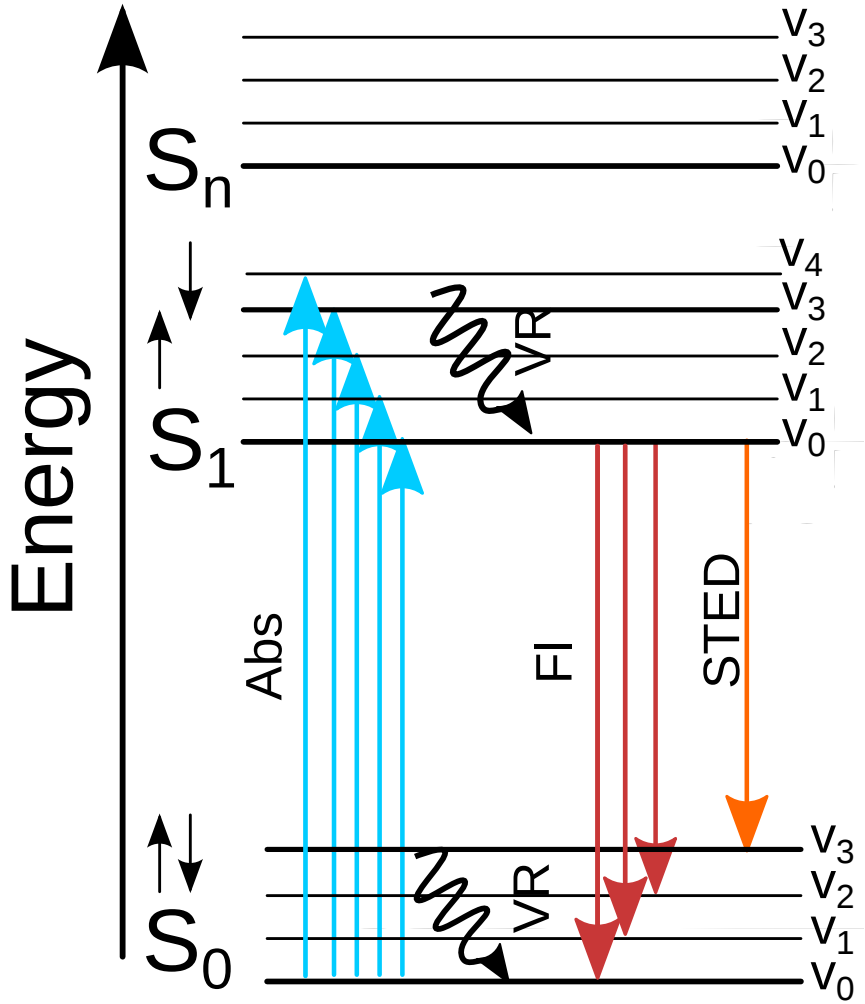
- Excitation: $S_0 V_0 \rightarrow S_1 V_k$, ground state into first excitation, short wavelength
- Stimulated emission: $S_1 V_0 \rightarrow S_0 V_n$. Ensure that λ_{STED} does not carry enough energy for excitation. Medium wavelength.
- Fluorescence: Emission $S_1 V_0 \rightarrow S_0 V_m$ with $m < n$. Filter out λ_{STED} with a sharp filter. Long wavelength.



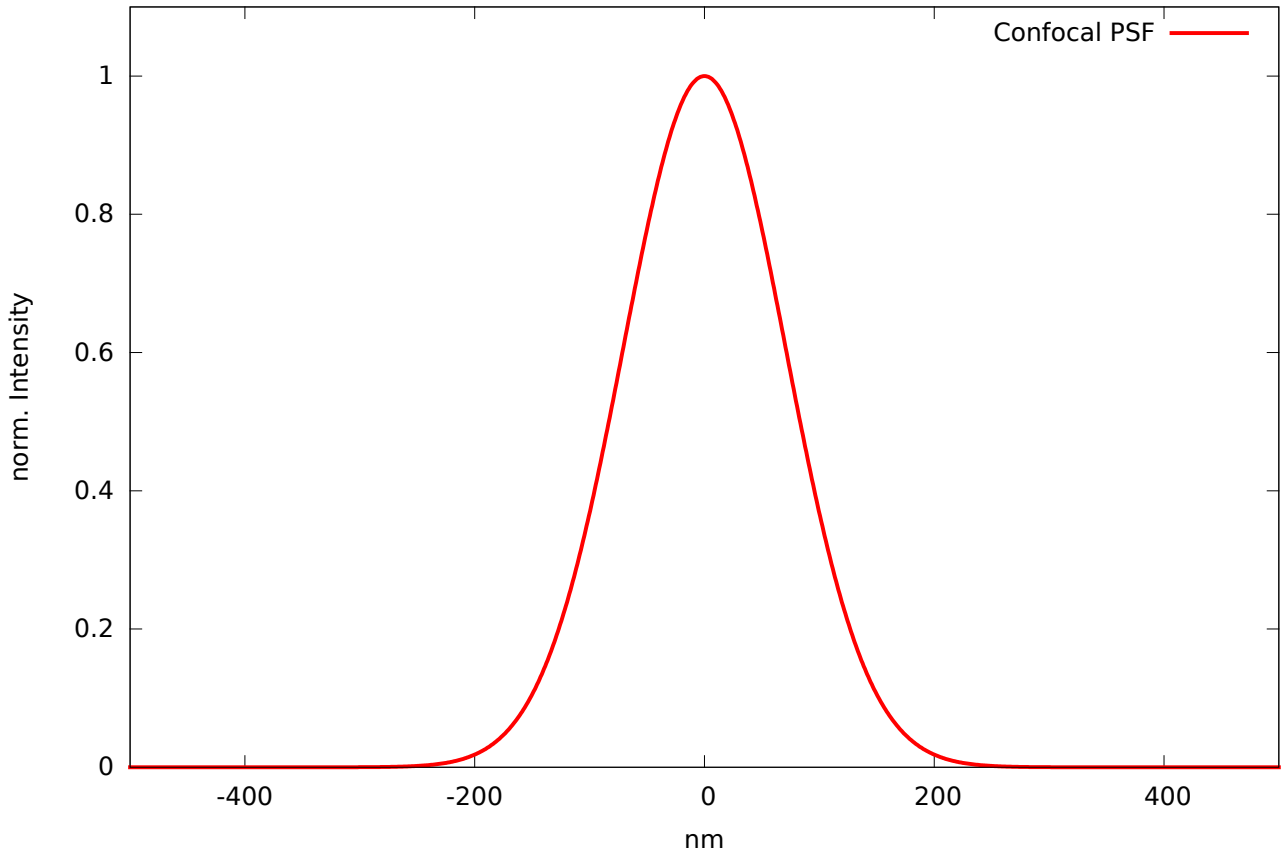
STED

Properties of λ_{STED}

- with rising intensity, probability shifts towards the STED-enhanced transmission
- this process is non-linear, with a cut-off at (almost) full probability on the STED transition
- Intensities depend on fluorophores (probability) spectrum
- only measure the fluorescence *not* at λ_{STED} (steep filters)

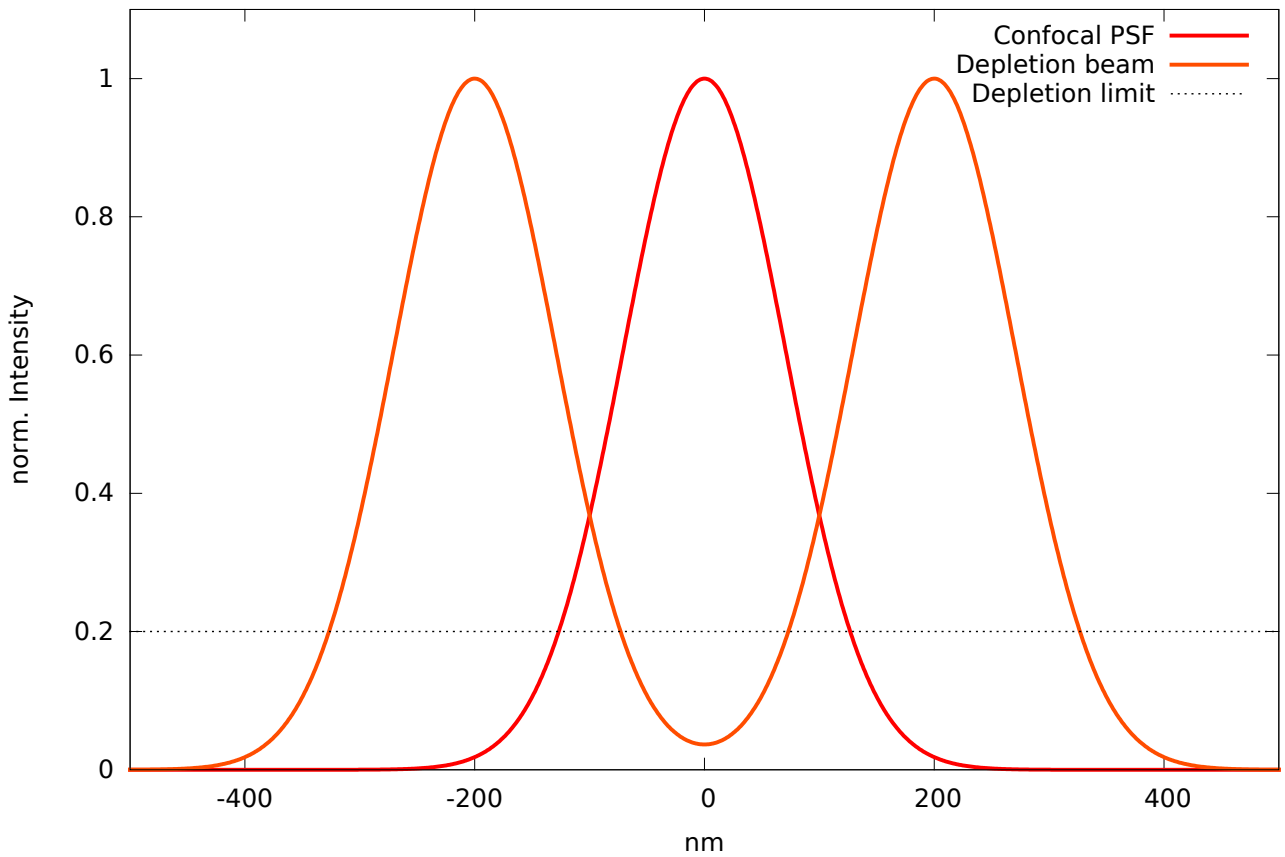


STED - PSF



PSF for standard confocal excitation / detection

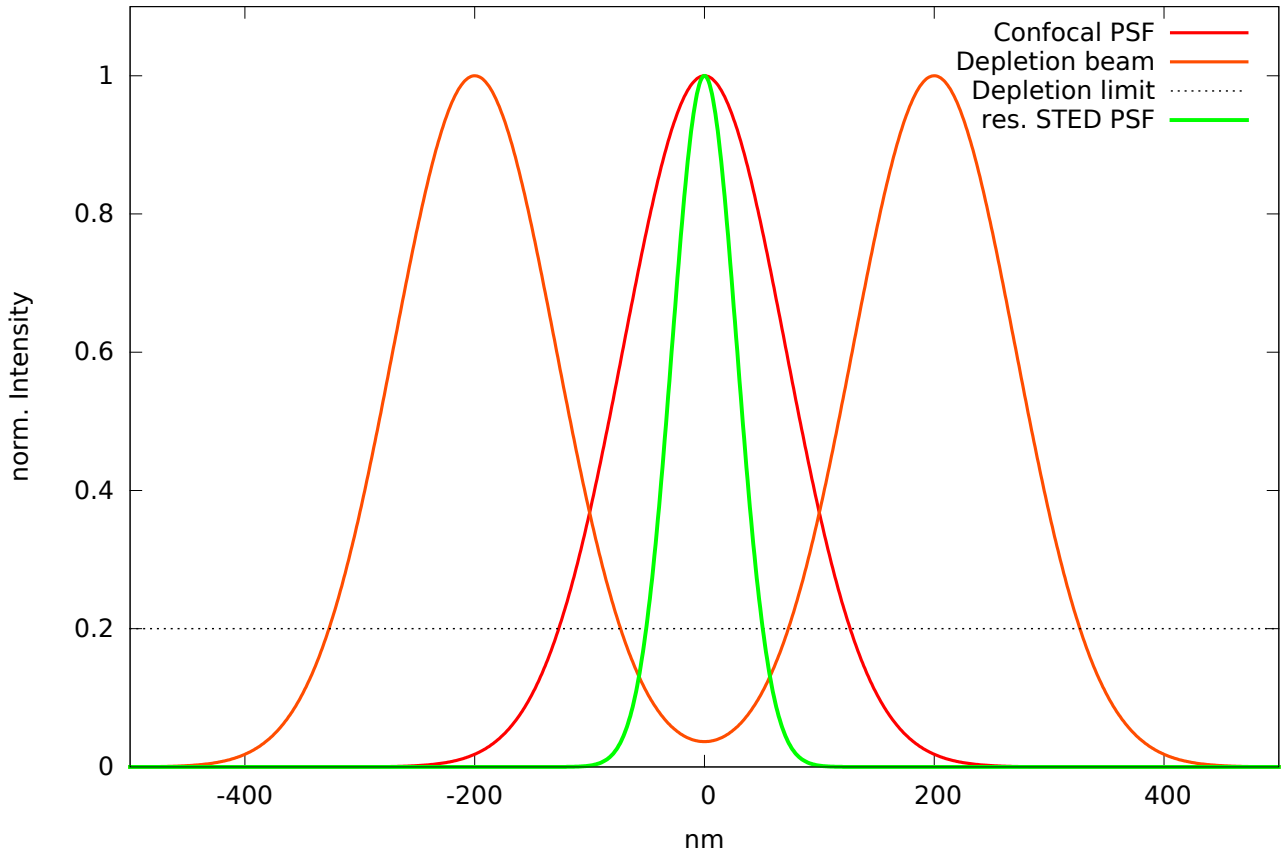
STED - PSF



Add a (shaped) depletion beam

- Intensities (between beams) are arbitrary
- Assume here: $\frac{1}{5}$ intensity yields full depletion

STED - PSF



Result / STED PSF:

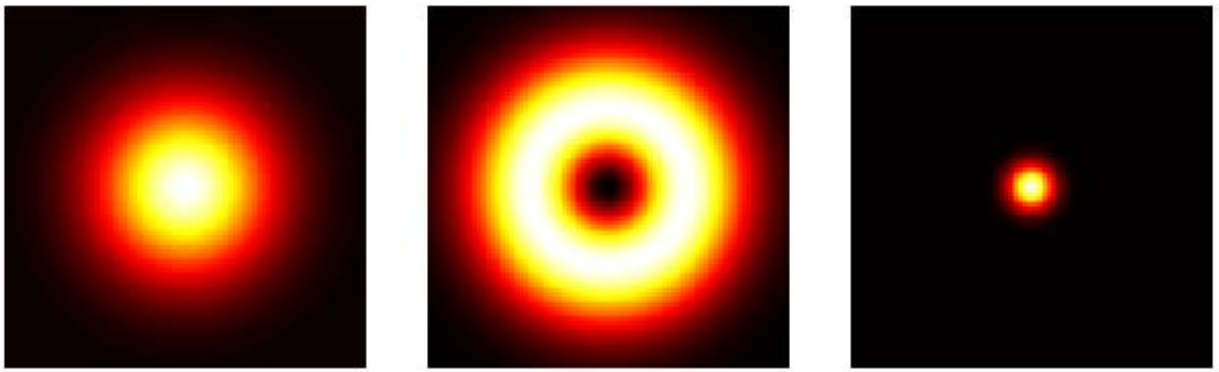
- Emission only for $I_{\text{ex.}} > 0$ *and* where $I_{\text{STED}} < 0.2$.
- STED PSF width now scales with I_{STED}.

STED - 2D PSF intensity profile

Resolution

$$d = \frac{\lambda}{2 \cdot \mathrm{NA} \cdot \sqrt{1 + \frac{I_{\mathrm{STED}}}{I_{\mathrm{sat.}}}}}$$

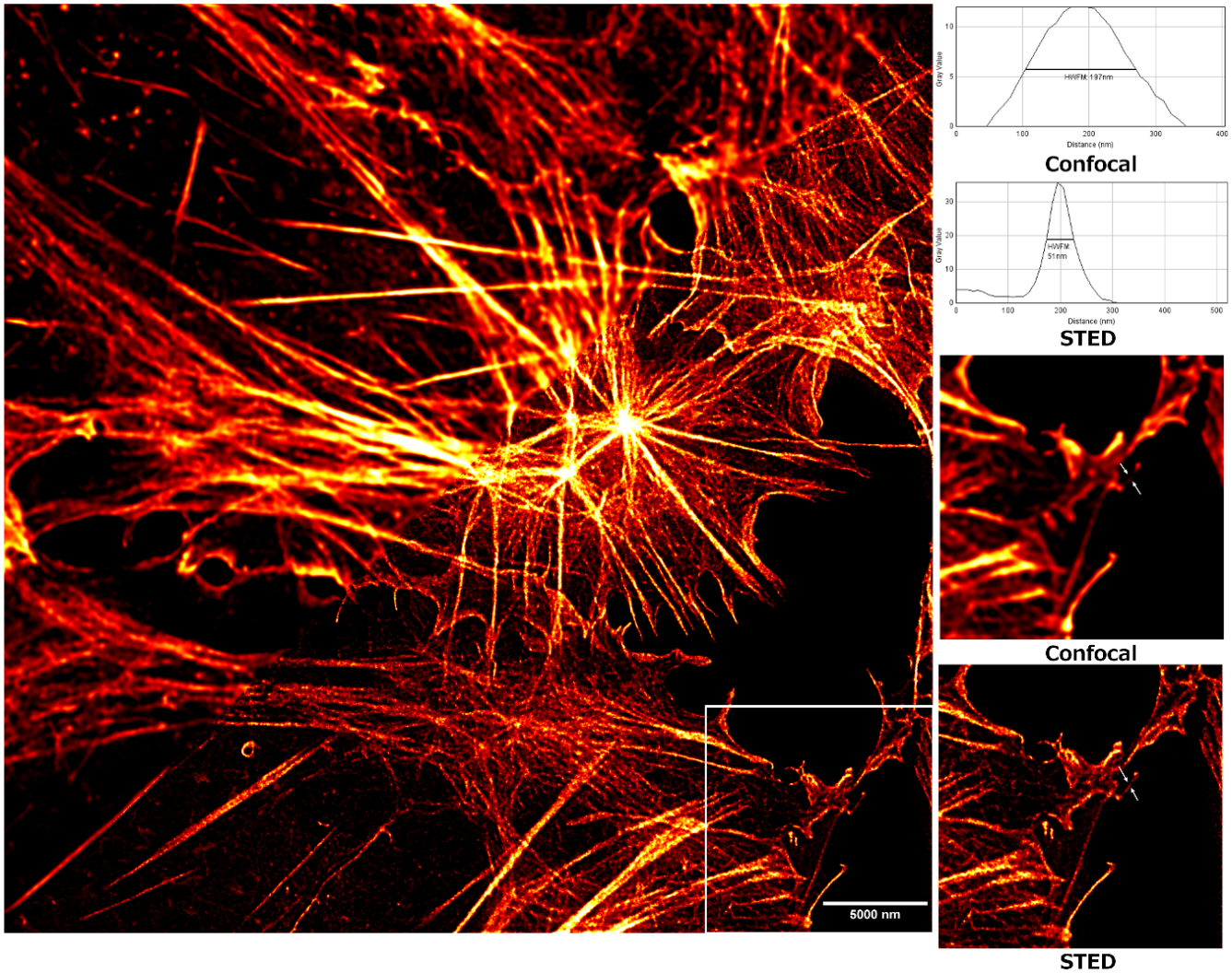
- Theory: No limit to resolution, just increase I_{STED}
- Reality: I_{STED} destroys the sample, even for modest resolutions
- 50nm for relevant samples, 2nm as proof-of-concept



Wikimedia / STED PSF

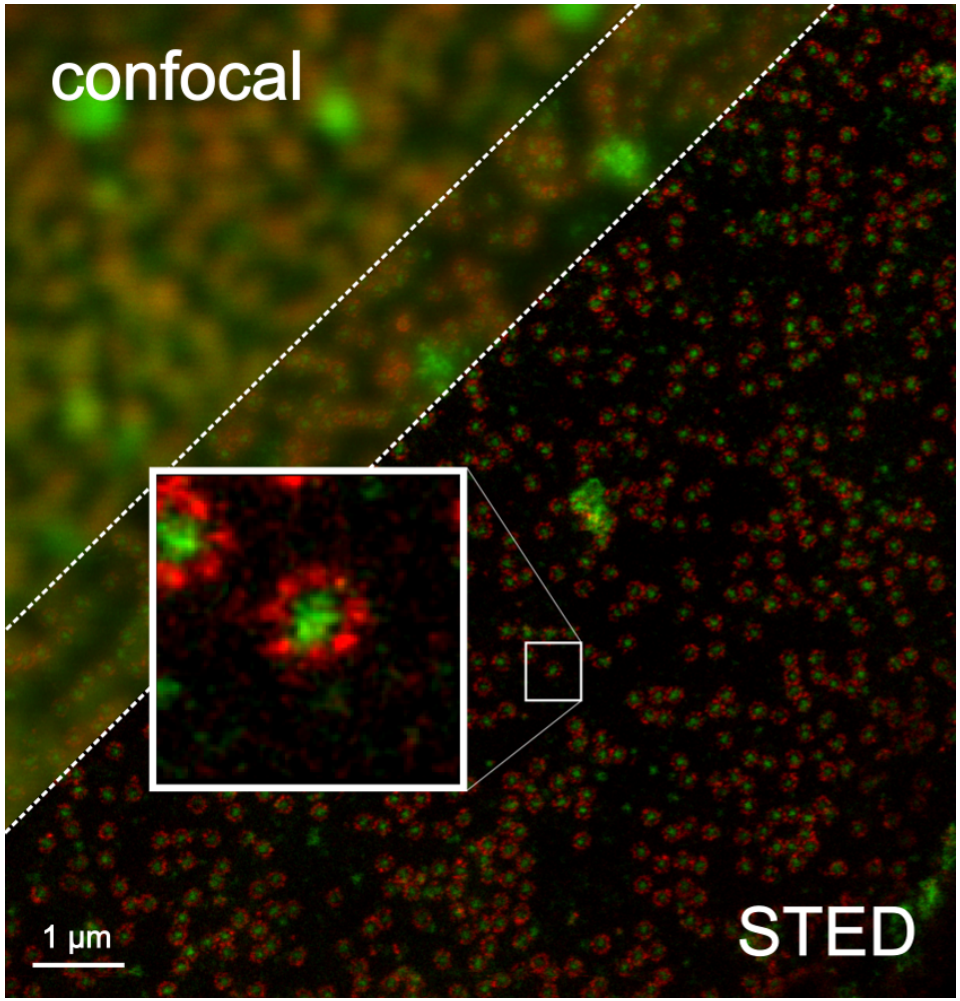
- Instrument: Confocal microscope with second STED beam
- Some diffraction element to form the doughnut

STED - Example (Actin)



Wikimedia / STED Actin 50nm

STED - Example (NPC)



Wikimedia / STED 2color NPC

STED-like techniques: RESOLFT

RESOLFT: **Re**versible **S**aturable **O**ptica**l** **F**luorescence **T**ransitions

GSD - ground-state depletion

- Same spatial beam layout as STED
- Instead of depletion, induce a transition to / from non-fluorescent triplet state: Switch the fluorophore on / off
- Slower (transition time), but less damaging (intensities)

RESOLFT with switchable dyes

- Same spatial beam layout as STED
- Proteins or organic dyes with switching wavelength
- Slower (even as GSD), photo-damage only dependent on dye switching properties

STED and RESOLFT - summary

- $\frac{1}{3}$ Nobel prize (Stefan Hell) awarded for STED
- First publications combine RESOLFT-like switching with SIM frequency shifting: Even higher resolution
- Instrumentation: Confocal microscope with optical add-on, minimal post-processing
- Drawback / Trade-offs: Speed, resolution, intensity / photo-damage.
- No STED in Bielefeld

Stochastic localization microscopy

Stochastic localization microscopy

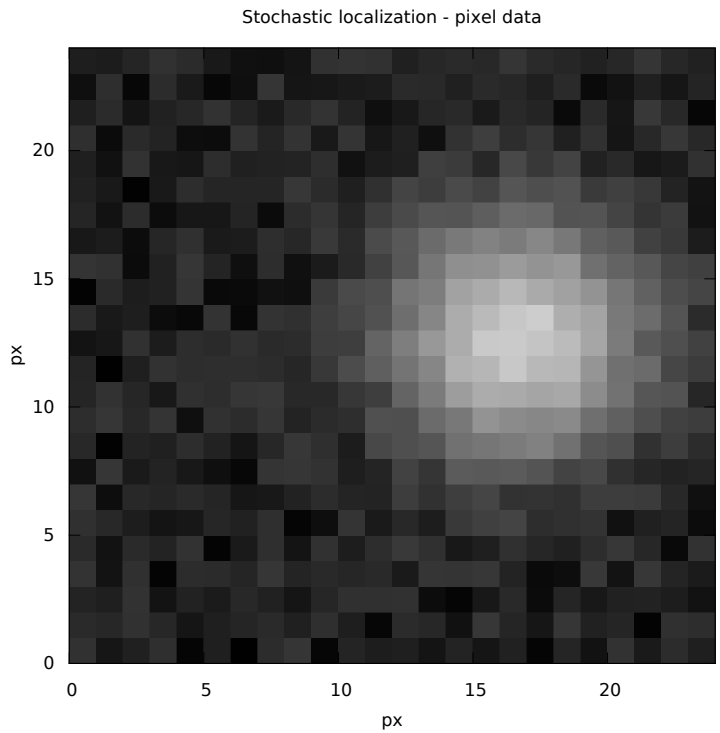
Idea

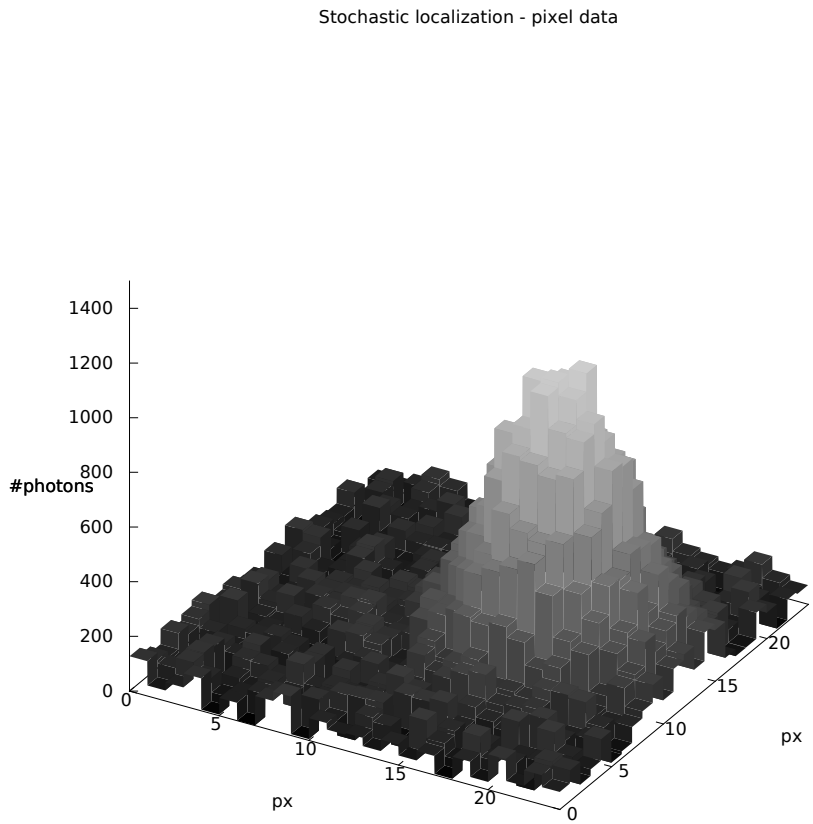
- Fluorophore: single molecule (sub-nanometer) emitting photons $\rightarrow$ point-source
- Photons distributed on sensor $\rightarrow$ Point-spread function
- Usual wide-field: overlap of all Fluorophore point-spread functions
- Idea: Observe a single fluorophore

Microscope

- Standard wide-field microscope (good NA)
- Sensitive camera: Events with $\sim 1,000 - 10,000$ photons
- Fast camera: Capture $\sim 2,000 - 50,000$ frames
- Multiple laser lines, some with high power
- All illumination modes (TIRF, HiLo, EPI)

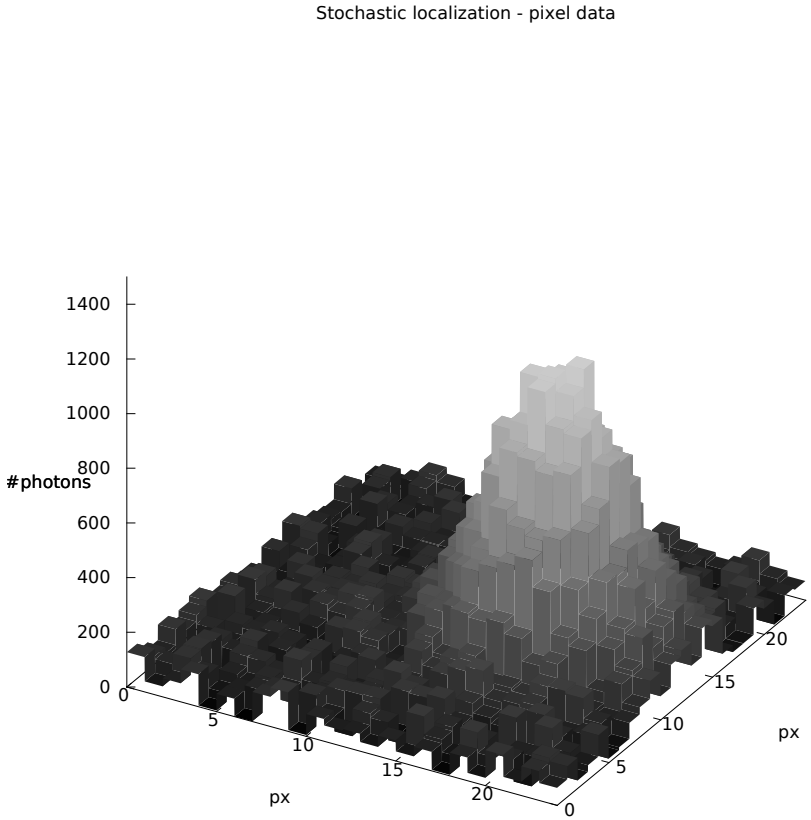
A single fluorophore

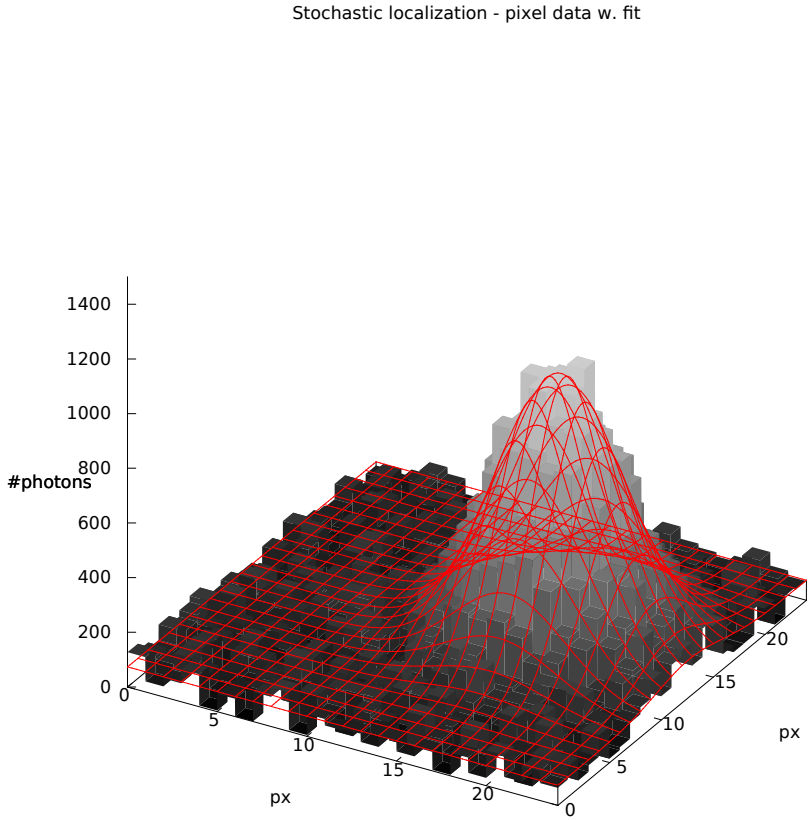




(simulated) intensity distribution, incl. photon shot noise
Reality: Larger pixels, less photons

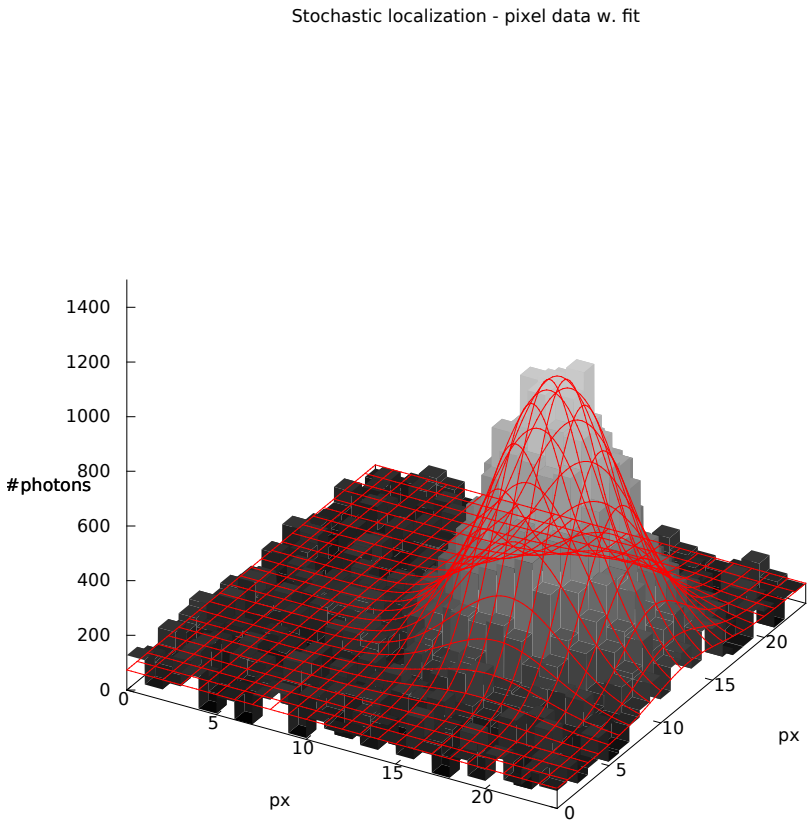
Fitting the distribution

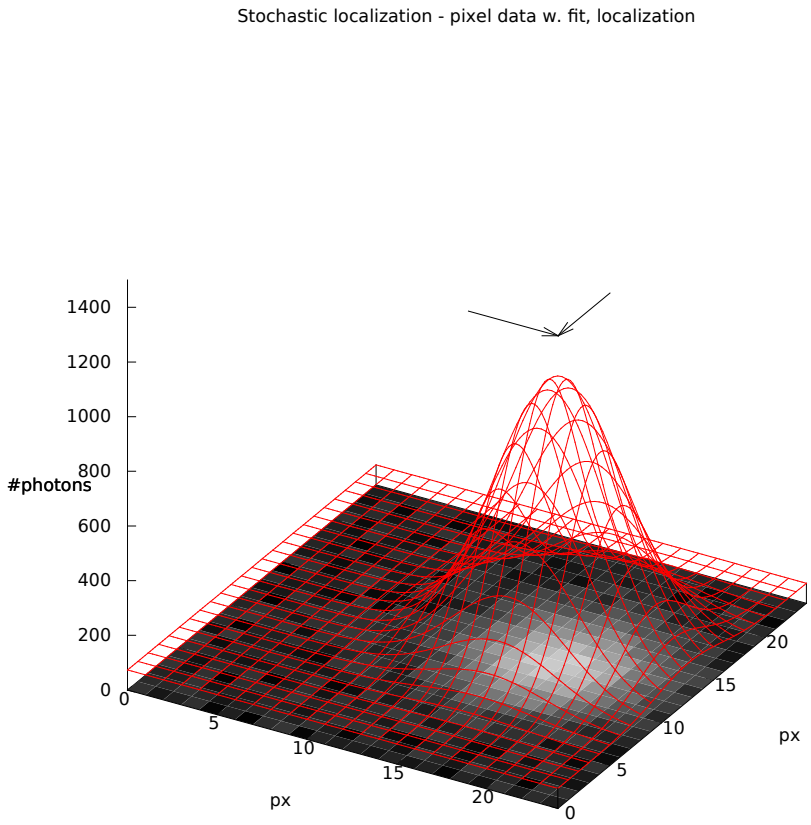




2D Gaussian fit to the emission

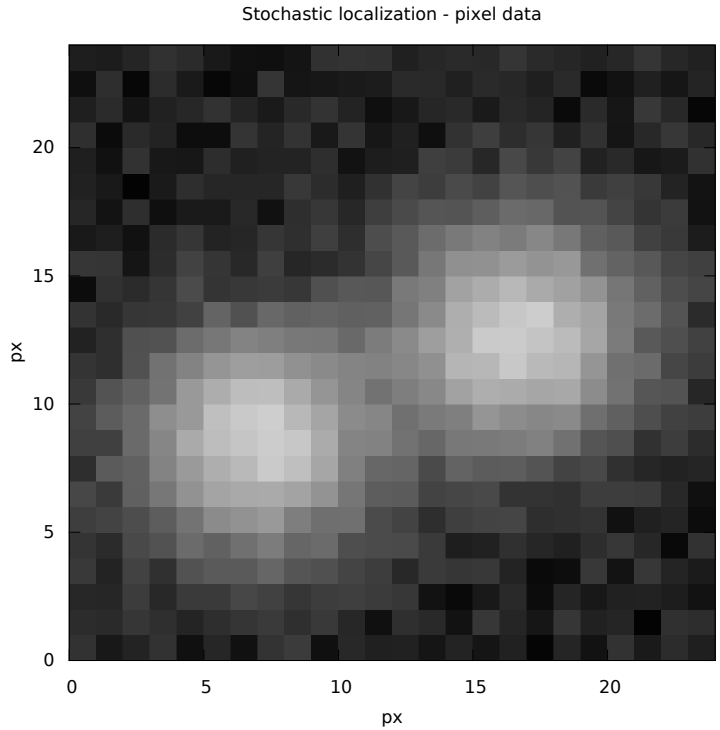
Fitting the distribution

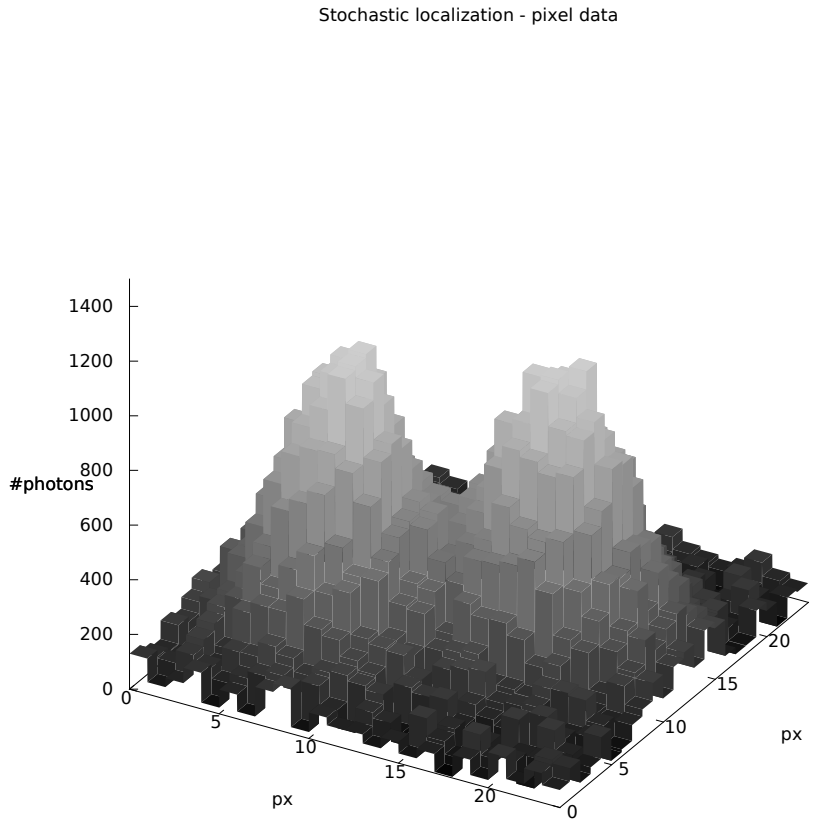




Fit yields: Position, Intensity, FWHM (all with fit precision)

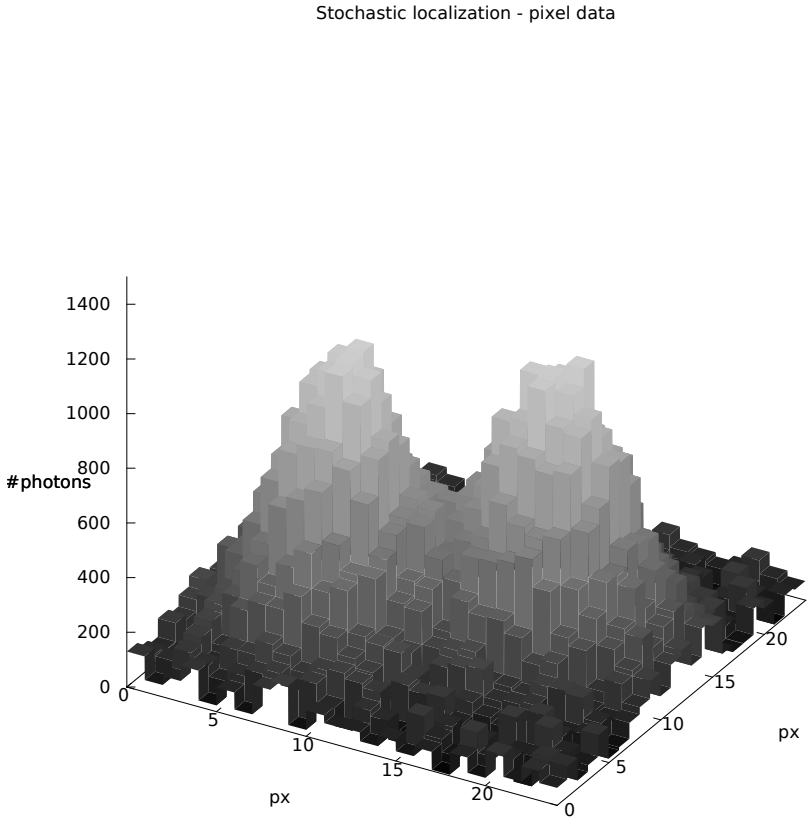
Two fluorophores with enough distance

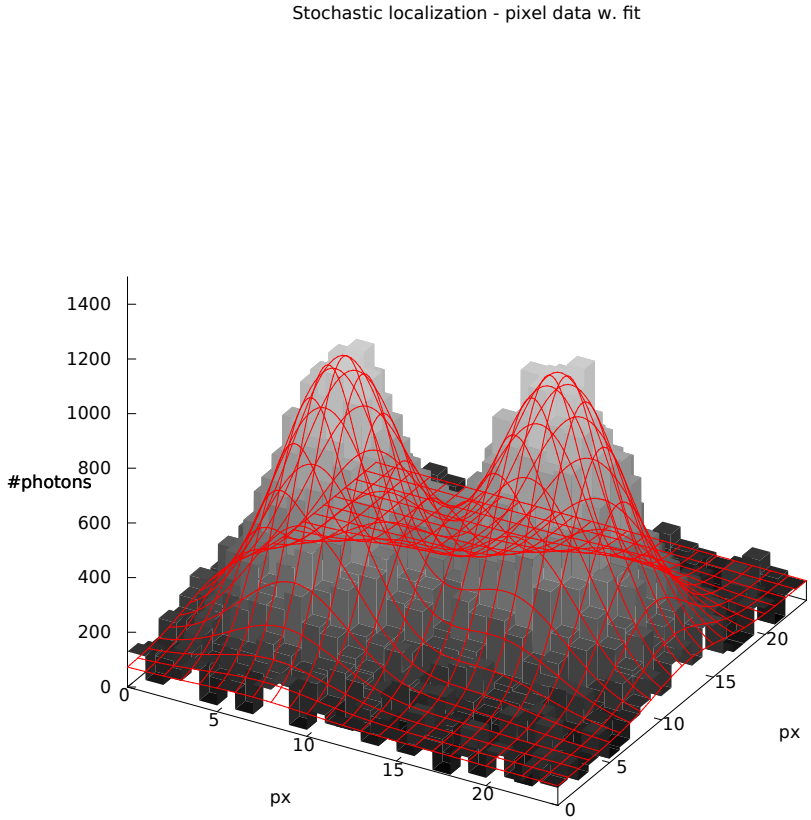




Emitter distance larger than FWHM

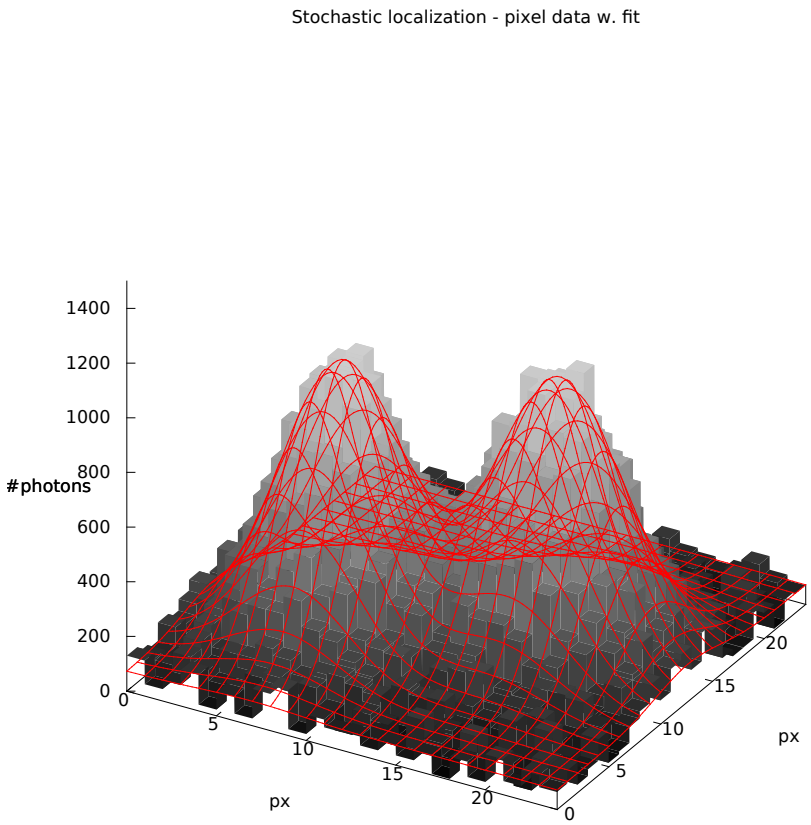
Fitting the distribution

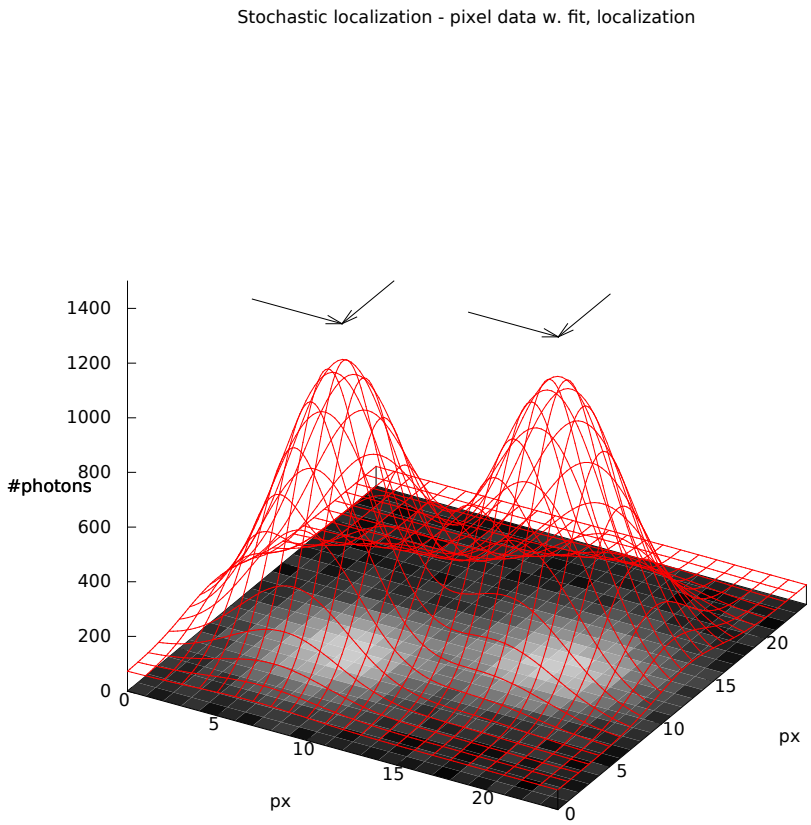




Gaussian fit still works

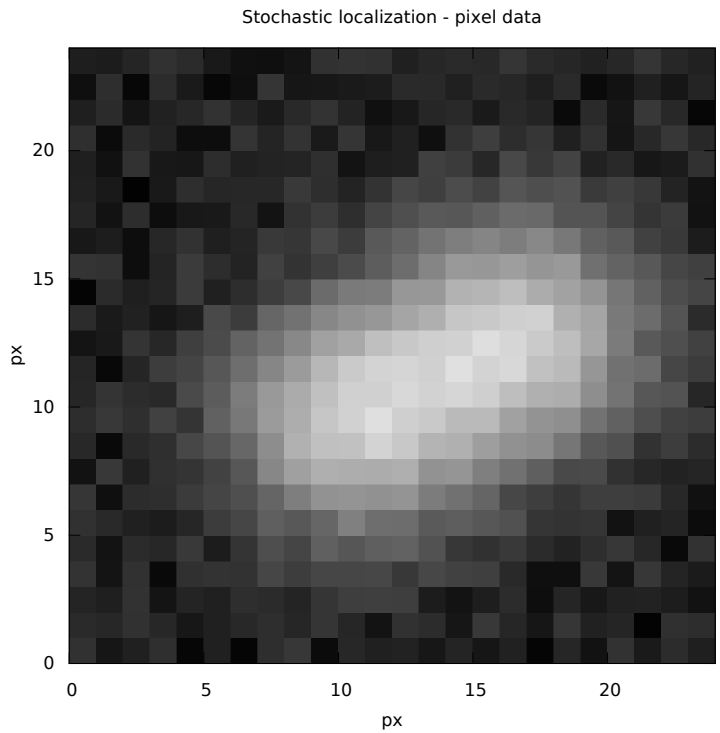
Fitting the distribution

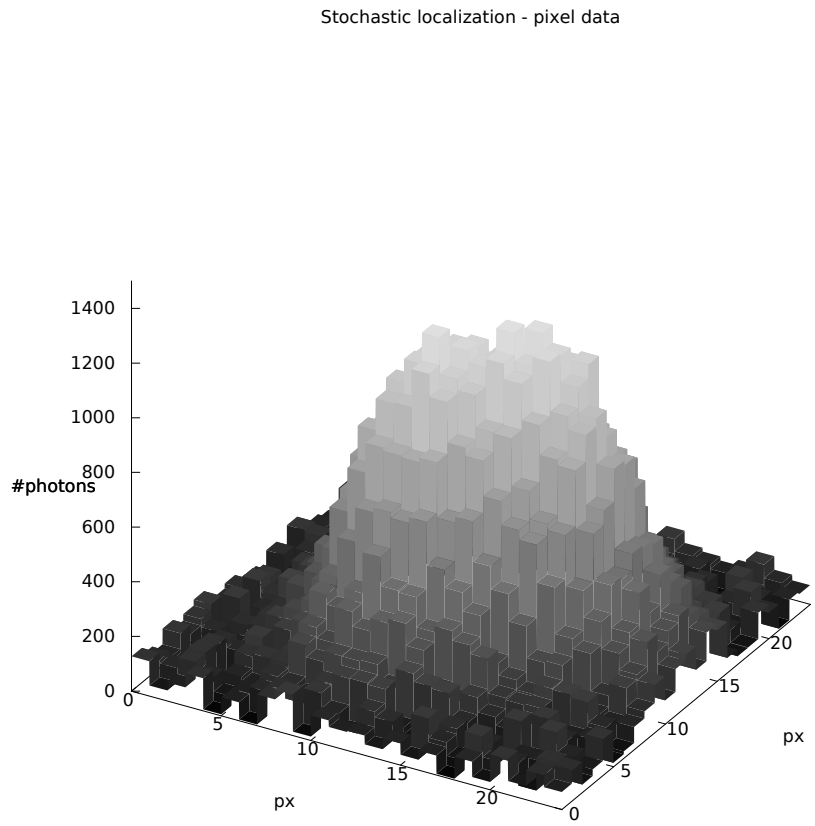




Position and intensity for each emitter

Fluorophores too close





Fluorophores within FWHM: fit failes

Analysing localization microscopy data

Algorithm

For each frame:

- Find possible emitters (e.g. intensity)
- Fit Gaussian distribution
- Reject errors (emitters to close,...)
- Store a long list, values and fit precision:
  - position
  - intensity
  - FWHM
  - frame number

(Fiji includes this as a plugin)

Next week:

- Sample preparation
- Resolution
- Visualization
- Extension to 3D