Nonliear structured-illumination microscopy via saturated SIM and photoswitchable protein

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Outline

Introduction: Resolution limit

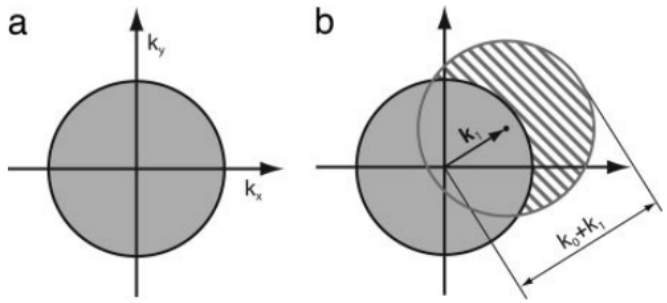
Resolution limit is a maximum spatial frequency k_0 that can be observed by microscope.

Resolution

For a light microscope, $\mathbf{k}_0 = \frac{2NA}{\lambda_{em}}$ where λ_{em} is the observation wavelength and NA is the numerical aperture of the objective lens.

In 2D frequency space, this limit defines a observable region

$$|\mathbf{k}| \leq \mathbf{k}_0$$



Introduction: Structured-illumination Microscopy

If the illumination contains a spatial frequency $\mathbf{k}_1$, then by moire fringes effect, the observable region (indirectly) will be

$$|\mathbf{k} - \mathbf{k}_1| \leq \mathbf{k}_0$$

Since new information can be extracted by phase shift method, the new information increases the highest observable spatial frequency (the resolution) from $\mathbf{k}_0$ to $\mathbf{k}_0 + \mathbf{k}1. \longrightarrow \max \mathbf{k}_1$

However: The set of spatial frequencies that can be generated in a light field is limited by diffraction in the same way as the set of frequencies that can be observed.

$$\mathbf{k}_1 \leq \frac{2NA}{\lambda_{exc}} \approx \mathbf{k}_0$$

Result in conventional SIM factor of 2.

Introduction: Non-linearity

The image we observed from microscope is modeled by

$$\boldsymbol{D}(\mathbf{x}) = (Em * P)(\mathbf{x})$$

where $\boldsymbol{D}$ is the distribution of photons on the detector,Em is the emission distribution of the sample, P is the PSF of microscope.
In conventional fluorescence microscopy, the emission rate responds linearly to the Illumination

$$Em(\boldsymbol{x}) = S(\boldsymbol{x}) \cdot I(\boldsymbol{x}),$$

where $S(\mathbf{x})$ is the sample fluorescence distribution.

Introduction: Non-linearity

A nonlinear photophysical process is that

$$Em(\boldsymbol{x}) = S(\boldsymbol{x}) \cdot F(I(\boldsymbol{x})),$$

where F describes the nonlinear behavior of the sample in response to the illumination light.By Fourier series expansion, we can fomulate the nonlinearity as

$$\begin{aligned} F[I(\mathbf{x})] =& b_0 + b_1 \cos\left(2\pi k_0 \mathbf{x} + \varphi\right) + b_2 \cos\left(4\pi k_0 \mathbf{x} + 2\varphi\right) \\ &+ b_3 \cos\left(6\pi k_0 \mathbf{x} + 3\varphi\right) + \ldots \end{aligned}$$

The observation in frequency space contains higher order harmonics:

$$\tilde{D}(\mathbf{k}) = \mathrm{OTF}(\mathbf{k}) \sum_{m=-\infty}^{\infty} b_m \tilde{S}\left(\mathbf{k} - m k_0\right) e^{-im\varphi}.$$

Introduction: Practical Limitation

- Theoretically , a non-polynomial nonlinear SIM can produce an infinite number of harmonics, corresponding to infinite resolution.
- In practice, the resolution is finite due to signal-to-noise ratio and photostability.

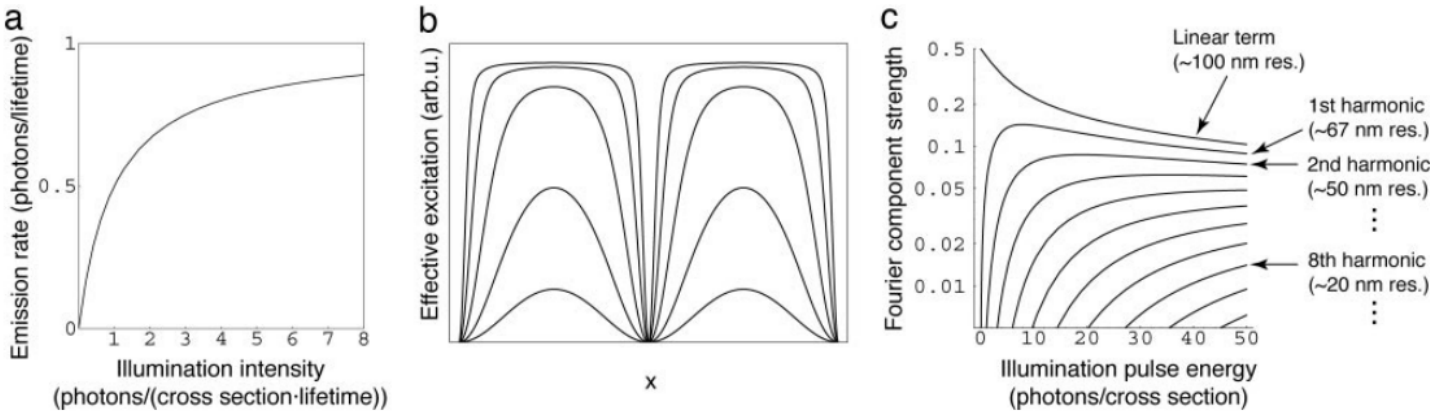
Outline

Saturated Non-linearity[1]

Here a particular nonlinear phenomenon: *saturation of the excited state* S_1 is discussed.

1. Firstly,a fluorophore molecule in its electronic ground state S_0 is excited to the first excited state S_1 by absorbing λ_{exc}.
2. The fluorophore molecule decays back to S_0 after an average time τ (the fluorescence lifetime) as it emits a photon at λ_{em}.
3. When the illumination intensities above one photon per absorption cross section, it cannot respond linearly since on average each molecule can emit at most one photon per τ.
4. If the sample is illuminated by a sinusoidal light pattern with a peak intensity that is near or above this threshold, then the pattern of emission rate per fluorophore takes on a nonsinusoidal shape.

Generation of harmonics by nonlinear fluorescence



1. The nonlinear dependence of the fluorescent emission rate on the illumination intensity in the saturation regime.
2. The emission pattern resulting from sinusoidally patterned illumination with peak pulse energy densities of (from the bottom to top curve) 0.25, 1, 4, 16, and 64 times the saturation threshold.
3. As the illumination energy increases, more and more harmonics come into play (lower curves).

Investigation on Non-linearity

Illumination photon flux I is applied to a fluorophore with absorption cross section σ,the emission rate E will increase exponentially with time t

$$E \propto \frac{I\sigma}{I\sigma + k_f}(1 - e^{-(I\sigma + k_f)t})$$

where $k_f = \frac{1}{\tau}$ is the decay rate

Equilibrium

For steady illumination or long pulses (pulse length $t_p >> \tau$), the intensity dependence of E is the rational function $\frac{I\sigma}{I\sigma + k_f}$.

Non-equilibrium

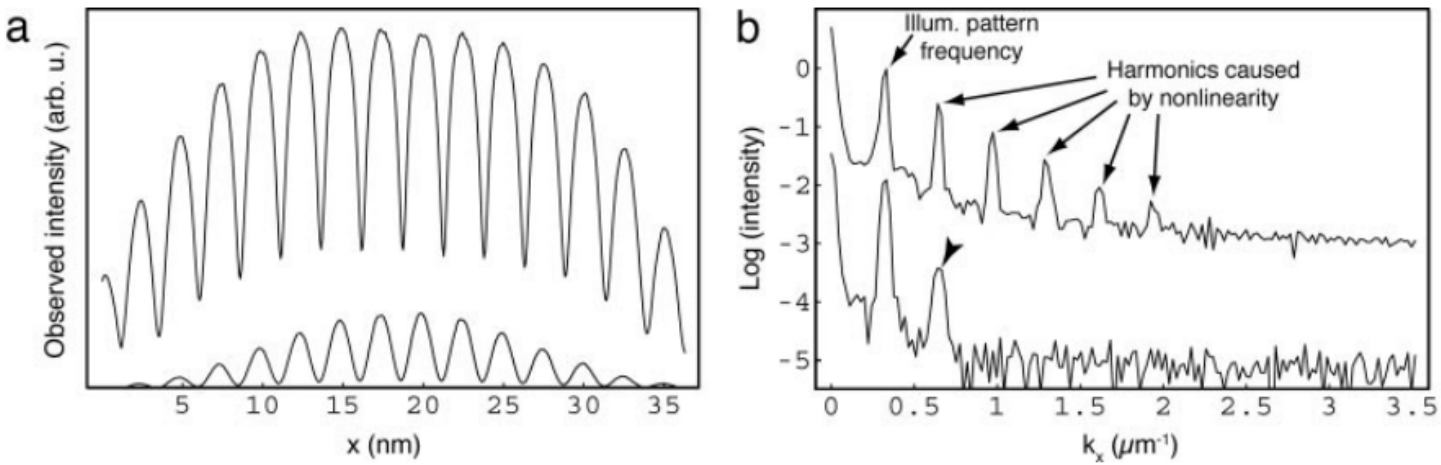
For short pulse length $t_p << \tau$, it produces an exponential dependence $E \propto \{1 - e^{-(I\sigma + k_f)t_p}\}$, which is somewhat more effective at generating harmonics.

For SSIM, we choose short pulse length 0.64 ns compared to $\tau = 2 \sim 4ns$.

Demonstration of Saturation

Demonstration of Experiment:

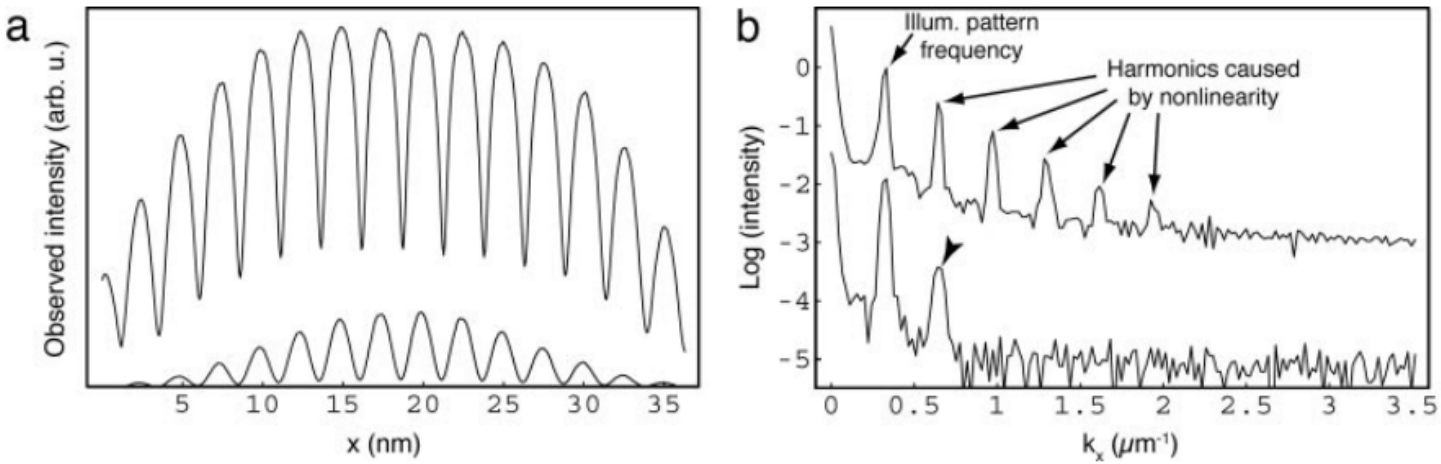
- A parallel pattern with period of $2.5\mu m$(coarser than resolution limit $0.19\mu m$) of parallel lines, was produced by passing the laser light through a transmission phase grating and projecting a demanified image of the grating onto the sample.
- By allowing only diffraction orders - 1 and + 1 from the grating to reach the sample, the line pattern was made purely sinusoidal since two beams intersected at an angle in the sample to produce a sinusoidal interference pattern.



Demonstration of Saturation

Demonstration of Experiment:

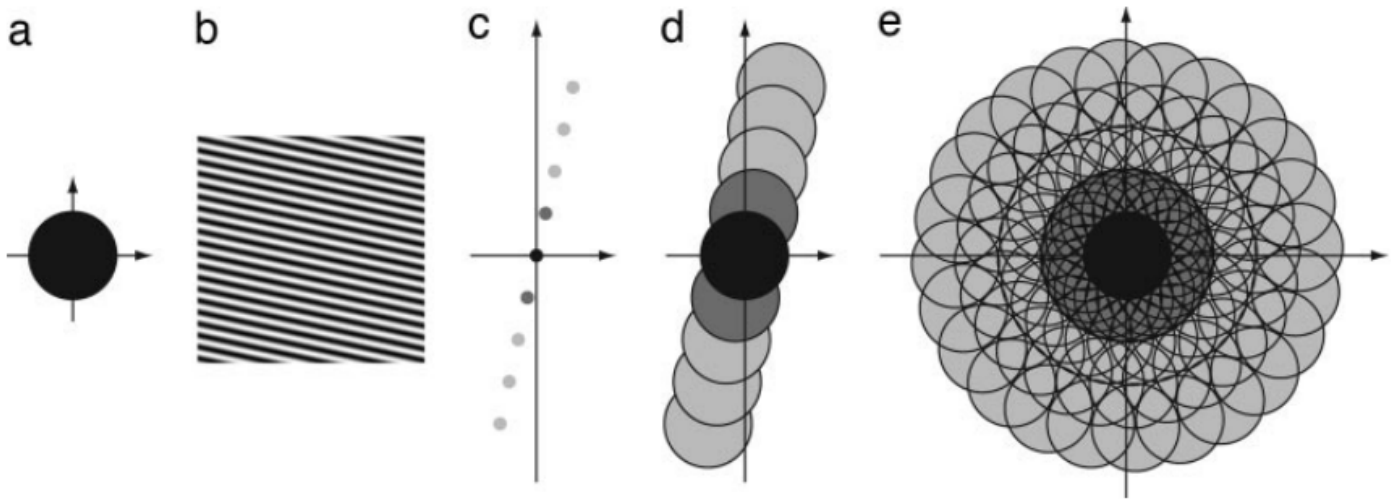
- At a relatively low peak energy density per pulse of $0.58mJ/cm^2$, the observed pattern of emission was approximately sinusoidal.
- At a higher peak energy density of $37mJ/cm^2$,however, the emission pattern changed drastically, taking on broad, flat peaks and sharp, narrow valleys.



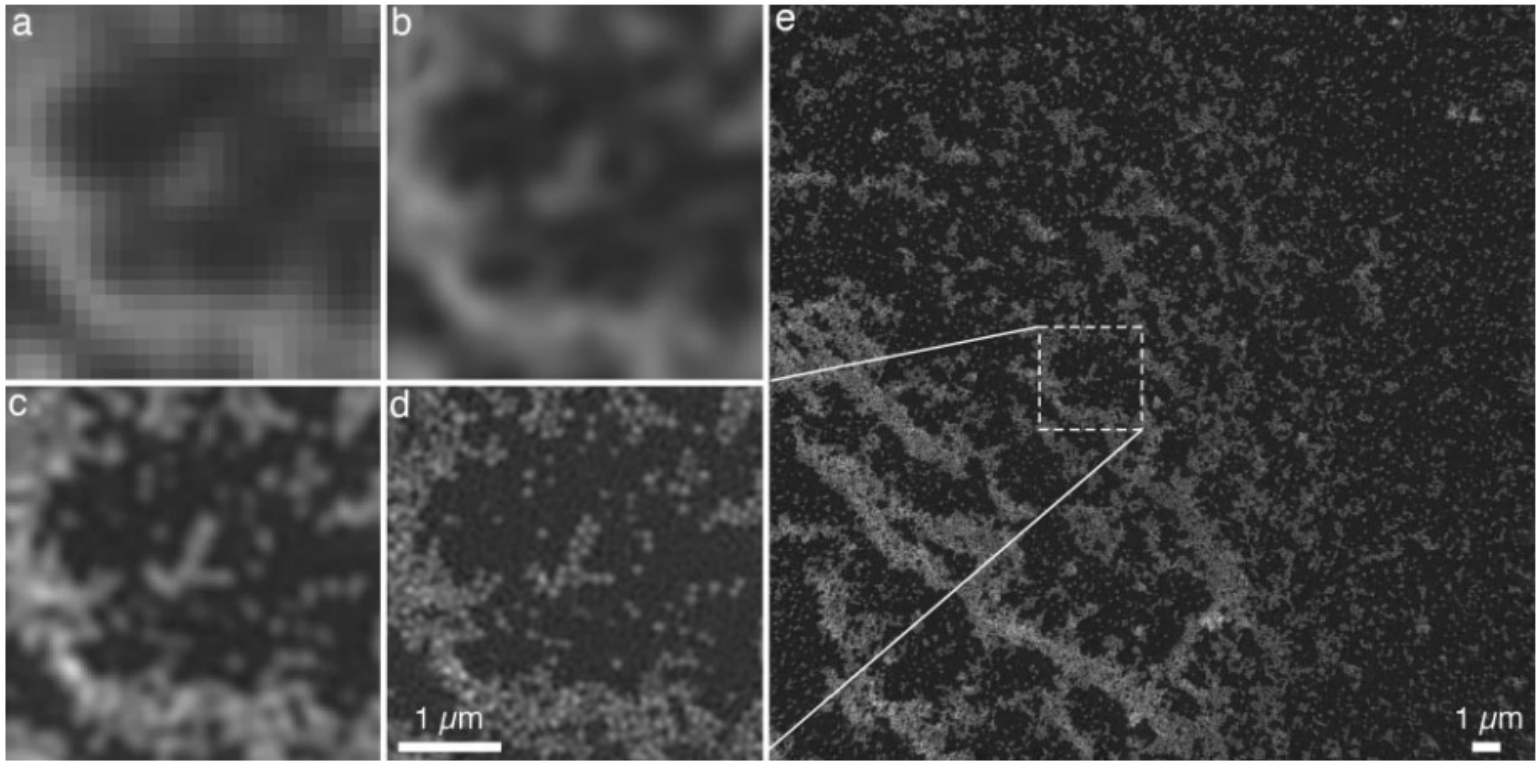
SSIM Resolution Test

- The illumination period of the resulting pattern was $0.20\mu m$, close to the theoretical limit of $0.19\mu m$ at this wavelength,
- The orientation and phase of this pattern were controlled by rotating and laterally translating the grating.
- Depositing 50 nm diameter fluorescent polystyrene microspheres on a cover glass and covering with a glycerol-based mounting medium.
- Pulse energy density of $5.3mJ/cm^2$ and an exposure time of 0.15 s
- The first three harmonics of the pattern were expected to be nonnegligible, corresponding to a total of nine superposed information components:$\Delta\phi = \frac{2\pi}{9}, \Delta\theta = \frac{\pi}{12}$

SSIM Resolution Test



SSIM Resolution Test



(a)is conventional microscopy; (b) is conventional microscopy with filtering (c) is conventional SIM (d) is SSIM.

Reconstruction Algorithm[2]

Here we consider a 2 order Nonlinear SIM case where 5 components is observable in one image.For orientation $\mathbf{p}_m$, the imaging model is

$$D_m(\omega) = O(\omega)H(\omega) + \frac{c_m^1}{2}e^{-i\phi}O(\omega - \mathbf{p}_m)H(\omega) + \frac{c_m^1}{2}e^{i\phi}O(\omega + \mathbf{p}_m)H(\omega)$$
$$+ \frac{c_m^2}{2}e^{-i2\phi}O(\omega - 2\mathbf{p}_m)H(\omega) + \frac{c_m^2}{2}e^{i2\phi}O(\omega + 2\mathbf{p}_m)H(\omega) + N$$

For a particular p_m ,five image of different phase $\phi^{(1)}, \phi^{(2)}, \phi^{(3)}, \phi^{(4)}, \phi^{(5)}$ is taken.

Outline

Reconstruction Algorithm

We have

$$\begin{bmatrix} D_{\phi^1,m} \\ D_{\phi^2,m} \\ D_{\phi^3,m} \\ D_{\phi^4,m} \\ D_{\phi^5,m} \end{bmatrix} = \begin{bmatrix} 1, \frac{c_m^1}{2}e^{-i\phi^1}, \frac{c_m^1}{2}e^{i\phi^1}, \frac{c_m^2}{2}e^{-i2\phi^1}, \frac{c_m^2}{2}e^{i2\phi^1} \\ 1, \frac{c_m^1}{2}e^{-i\phi^2}, \frac{c_m^1}{2}e^{i\phi^2}, \frac{c_m^2}{2}e^{-i2\phi^2}, \frac{c_m^2}{2}e^{i2\phi^2} \\ 1, \frac{c_m^1}{2}e^{-i\phi^3}, \frac{c_m^1}{2}e^{i\phi^3}, \frac{c_m^2}{2}e^{-i2\phi^3}, \frac{c_m^2}{2}e^{i2\phi^3} \\ 1, \frac{c_m^1}{2}e^{-i\phi^4}, \frac{c_m^1}{2}e^{i\phi^4}, \frac{c_m^2}{2}e^{-i2\phi^4}, \frac{c_m^2}{2}e^{i2\phi^4} \\ 1, \frac{c_m^1}{2}e^{-i\phi^5}, \frac{c_m^1}{2}e^{i\phi^5}, \frac{c_m^2}{2}e^{-i2\phi^5}, \frac{c_m^2}{2}e^{i2\phi^5} \end{bmatrix} \begin{bmatrix} O(\omega) \\ O(\omega - p_m) \\ O(\omega + p_m) \\ O(\omega - 2p_m) \\ O(\omega + 2p_m) \end{bmatrix}$$

For the non-singularity, $\phi^{(1)}, \phi^{(2)}, \phi^{(3)}, \phi^{(4)}, \phi^{(5)}$ differs by $\frac{2\pi}{5}$ each other.

Reconstruction Algorithm

Denote a phase shift component:

$$\begin{bmatrix} S_0(\omega) \\ S_{-1}(\omega) \\ S_{+1}(\omega) \\ S_{-2}(\omega) \\ S_{+2}(\omega) \end{bmatrix} = \begin{bmatrix} O(\omega)H(\omega) \\ O(\omega - p_1)H(\omega)\frac{c_1^1}{2}e^{-i\phi_1} \\ O(\omega + p_1)H(\omega)\frac{c_1^1}{2}e^{i\phi_1} \\ O(\omega - 2p_1)H(\omega)\frac{c_1^1}{2}e^{-i2\phi_1} \\ O(\omega + 2p_1)H(\omega)\frac{c_1^1}{2}e^{-i2\phi_1} \end{bmatrix}$$

By taking
$(\phi^{(1)}, \phi^{(2)}, \phi^{(3)}, \phi^{(4)}, \phi^{(5)}) = (\phi^{(1)}, \phi^{(1)} + \frac{2\pi}{5}, \phi^{(1)} + \frac{4\pi}{5}, \phi^{(1)} + \frac{6\pi}{5}, \phi^{(1)} + \frac{8\pi}{5})$

$$\begin{bmatrix} S_0(\omega) \\ S_{-1}(\omega) \\ S_{+1}(\omega) \\ S_{-2}(\omega) \\ S_{+2}(\omega) \end{bmatrix} = \begin{bmatrix} 1 & ,1 & ,1 & ,1 & ,1 \\ 1 & ,e^{-\frac{2\pi}{5}} & ,e^{\frac{2\pi}{5}} & ,e^{-\frac{4\pi}{5}} & ,e^{\frac{4\pi}{5}} \\ 1 & ,e^{-\frac{4\pi}{5}} & ,e^{\frac{4\pi}{5}} & ,e^{-\frac{8\pi}{5}} & ,e^{\frac{8\pi}{5}} \\ 1 & ,e^{-\frac{6\pi}{5}} & ,e^{\frac{6\pi}{5}} & ,e^{-\frac{12\pi}{5}} & ,e^{\frac{12\pi}{5}} \\ 1 & ,e^{-\frac{8\pi}{5}} & ,e^{\frac{8\pi}{5}} & ,e^{-\frac{16\pi}{5}} & ,e^{\frac{16\pi}{5}} \end{bmatrix}^{-1} \begin{bmatrix} D_{\phi^1,1} \\ D_{\phi^2,1} \\ D_{\phi^3,1} \\ D_{\phi^4,1} \\ D_{\phi^5,1} \end{bmatrix}$$

Determination of Parametes

As we observed that $S_0(\omega) = O(\omega)H(\omega), S_{+1}(\omega) = O(\omega + \mathbf{p}_1)H(\omega)\frac{c_1^1}{2}e^{i\phi_1}$, the object is to maximize the cross correlation of these two:

$$\mathbf{p}_1 = \arg\max_{p'} \sum_{\omega} S_0(\omega) S_1(\omega - p')$$

Also this can be extend to

$$\mathbf{p}_1 = \arg\max_{p'} \sum_{\omega} S_0(\omega) S_2(\omega - 2p')$$

Determination of Parametes

Another observation is that

$$\frac{S_{+1}(\omega - p_1)}{S_0(\omega)} = \frac{O(\omega)H(\omega - p_1)}{O(\omega)H(\omega)} \frac{c_1^1}{2} e^{-i\phi^1}$$

Thus

$$\frac{H(\omega)S_{+1}(\omega - p_1)}{H(\omega - p_1)S_0(\omega)} = \frac{c_1}{2} e^{-i\phi_1}$$

Also

$$\frac{S_{+2}(\omega - p_1)}{S_{+1}(\omega)} = \frac{O(\omega + p_1)H(\omega - p_1)\frac{c_1^2}{2} e^{-i2\phi_1}}{O(\omega + p_1)H(\omega)\frac{c_1^1}{2} e^{-i\phi_1}}$$

Then

$$\frac{H(\omega + p_1)S_{+2}(\omega - p_1)}{H(\omega - p_1)S_{+1}(\omega)} = \frac{c_1^2}{c_1^1} e^{-i\phi_1}$$

Then c_1^2 was determined.

Shifting Frequency Components to True Position

After tuning the parameter, the identified components $\begin{bmatrix} S_0(\omega) \\ S_{-1}(\omega) \\ S_{+1}(\omega) \\ S_{-2}(\omega) \\ S_{+2}(\omega) \end{bmatrix}$ is shifted by

Fourier shift theorem to

$$\begin{bmatrix} \hat{S}_0(\omega) \\ \hat{S}_{-1}(\omega) \\ \hat{S}_{+1}(\omega) \\ \hat{S}_{-2}(\omega) \\ \hat{S}_{+2}(\omega) \end{bmatrix} = \begin{bmatrix} O(\omega)H(\omega) \\ O(\omega)H(\omega+p_1)\frac{c_1^1}{2}e^{-i\phi_1} \\ O(\omega)H(\omega-p_1)\frac{c_1^1}{2}e^{i\phi_1} \\ O(\omega)H(\omega+p_2)\frac{c_1^1}{2}e^{-i2\phi_1} \\ O(\omega)H(\omega-p_2)\frac{c_1^1}{2}e^{-i2\phi_1} \end{bmatrix}$$

For example:

$$\mathcal{F}\left[\mathcal{F}^{-1}\left\{\tilde{S}_u\left(\mathbf{k}-\mathbf{p}_\theta\right)\right\}\times e^{-i2\pi(\mathbf{p}_\theta\cdot\mathbf{r})}\right]=\tilde{S}_s\left(\mathbf{k}-\mathbf{p}_\theta\right)$$
$$\mathcal{F}\left[\mathcal{F}^{-1}\left\{\tilde{S}_u\left(\mathbf{k}+\mathbf{p}_\theta\right)\right\}\times e^{+i2\pi(\mathbf{p}_\theta\cdot\mathbf{r})}\right]=\tilde{S}_s\left(\mathbf{k}+\mathbf{p}_\theta\right)$$

Wiener Filter For Merging Components

The Approximate generalized Wiener Filter is given by

$$\tilde{O}(\omega) = \frac{\sum_{m,j} H^*(\omega + jp_m) \frac{\hat{S}_{m,j}}{c_{\hat{m},j}}}{\sum_{m,j} |H(\omega + jp_m)|^2 + \alpha^2} A\omega, \quad m = 1, 2, \ldots; j = 0, -1, +1, -2, +2$$

where we assume that the average power spectrum of noisy $\tilde{S}(\mathbf{k})\tilde{H}(\mathbf{k})$ is $\tilde{S}(\mathbf{k})\tilde{H}(\mathbf{k}) = |\tilde{H}(\mathbf{k})|^2 \mathcal{A}^2 |\mathbf{k}|^{-2\alpha} + \Psi_{0,\theta}$, where $\mathcal{A}, \alpha$ are constant estimate by nonlinear regression.
The average noise power $\Psi_{0,\theta}$in $\tilde{S}(\mathbf{k})\tilde{H}(\mathbf{k})$ is estimated by averaging the square of frequency amplitude of noisy $\tilde{S}(\mathbf{k})\tilde{H}(\mathbf{k})$ over frequencies k lying outside OTF support, where signal power is zero.

Outline

Introduction

- Saturation requires extremely high light intensities that are likely to accelerate photo-bleaching and damage even fixed tissue, this implementation is of limited use for studying biological samples.
- To realized such nonlinearity, reversible photoswitching of a fluorescent protein provides the required nonlinearity at light intensities six orders of magnitude lower($1 - 10W/cm^2$) than those needed for saturation($1mW/cm^2$)

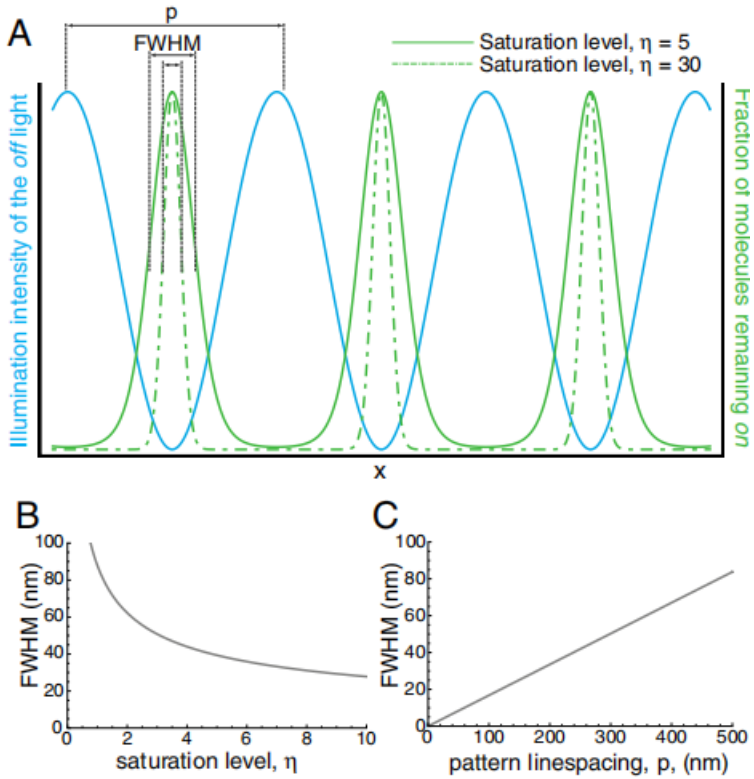
Introduction to Photo-switchable Fluorescent[3]

Photoswitching is an inherently nonlinear process and has been proposed as an alternative to saturation or stimulated emission.

1. Photo-switchable fluorescent molecules can be reversibly switched between two spectrally distinct states using light; saturating either of these population states results in a nonlinear relationship between the fluorescence emission and the illumination intensity.
2. Dronpa can be reversibly switched between a fluorescent on state with an excitation peak at 503 nm and a nonfluorescent off state with an absorption peak at 390 nm using light.
3. Saturating the off state should result in cleaner minima of the illumination pattern leading to higher signal-to-noise ratio of the higher-order harmonics.

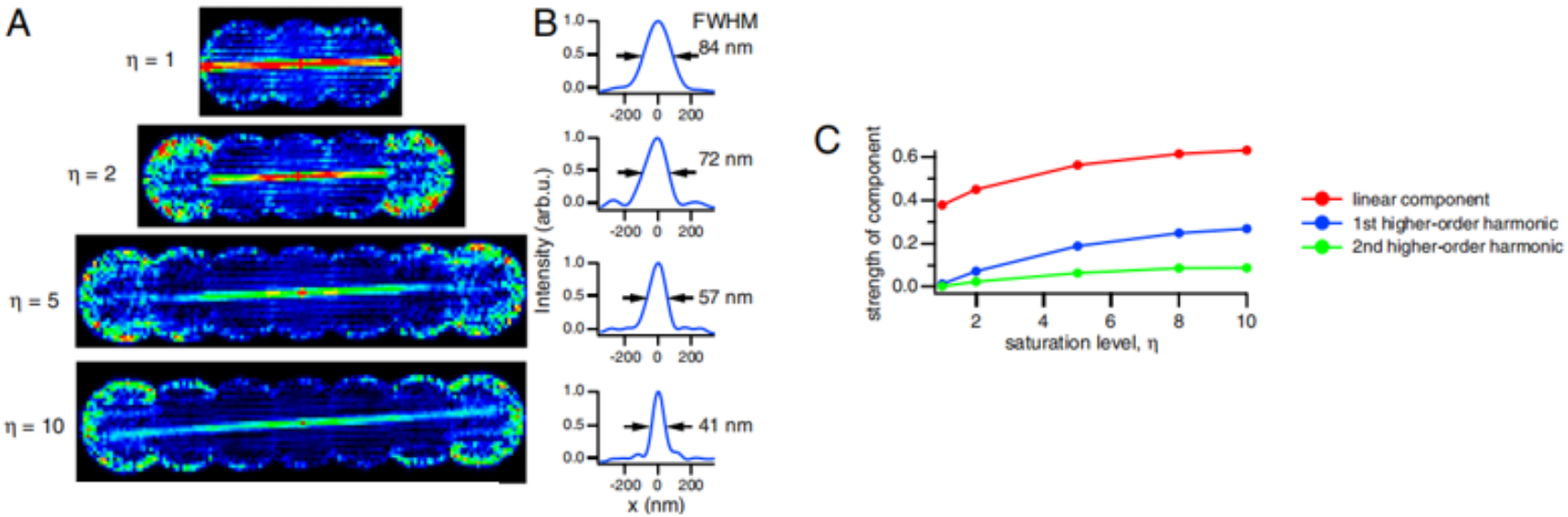
Introduction to Photo-switchable Fluorescent

Using blue light $488nm$ illumination,Dronpa decays to its off state at a characteristic timescale η_{off}(dependent on intensity).The saturation level $\eta = \tau_{exp}/\tau_{off}$.Using a sinusoidal pattern of light to drive the molecules to their off state ,only molecules at the minima of the pattern will remain on.The on-state region gets confined as η increases.



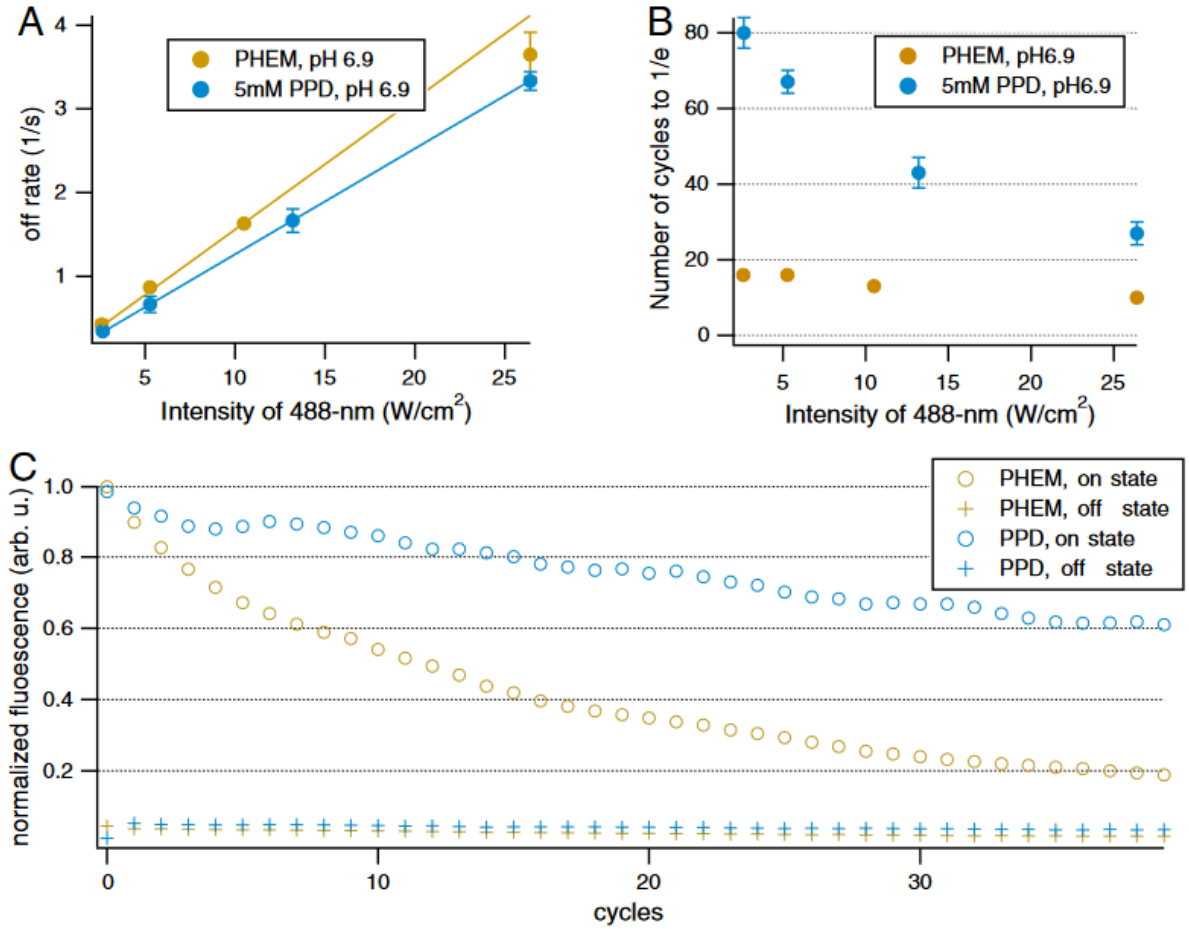
Resolution Dependencies

The number of contributing information components, is in principle infinite, there will only be a finite number that are detectable above the noise.The saturation level affects the number of these detectable higher-order harmonics and this in turn determines the final NL-SIM resolution.



Resolution Dependencies

When using photoswitching, each image corresponds to a photo-switching cycle, and thus a photoswitchable molecule able to withstand multiple rounds of cycling before photobleaching is needed.



NL-SIM with Photoswitchable Moleculars

Each image used during processing is a result of three exposures.

- **1. Turn on:** We use an activation wavelength light to turn on the sample uniformly.
- **2. Turn off:**Using a one-dimensional sinusoidally varying pattern of light that drives the molecules to the off state

$$I_{off} = \frac{I_0}{2}(1 - \cos(2\pi k_0 \mathbf{x} + \phi))$$

After some time T, the distribution of on-state molecules will be

$$S_{on}(x) = e^{-\frac{T}{I_0 \tau_{off}} I_{off}(x)} S(x)$$

We use the saturation level $\eta = \frac{T}{I_0 \tau_{off}} \frac{I_0}{2}$ to denote the constant effect.

NL-SIM with Photoswitchable Moleculars

Each image used during processing is a result of three exposures.

- **3: Expose by uniform illumination**

$$Em(\mathbf{x})I_0 \cdot S_{on}(\mathbf{x}) = I_0 e^{-\frac{\eta}{I_0} I_o f f(x)} = I_0 e^{-\frac{\eta}{2}(1-\cos(2\pi k_0 \mathbf{x}+\phi))}$$

The corresponding nonlinear response is

$$F[I_{off}(\mathbf{x})] = e^{-\frac{\eta}{I_0} I_{off}(\mathbf{x})} I_0$$

Using approximation $1-\cos(2\pi k_0 \mathbf{x}+\phi)) \approx \frac{(2\pi k_0 \mathbf{x}+\phi)^2}{4}$ we observe that the emission is a Gaussian distribution around $2\pi k_0 \mathbf{x} + \phi = 0$

$$Em(\mathbf{x}) \approx e^{-\frac{\eta}{4}(2k_0 \pi \mathbf{x}+\phi)^2} I_0 S(\mathbf{x})$$

NL-SIM with Photoswitchable Moleculars

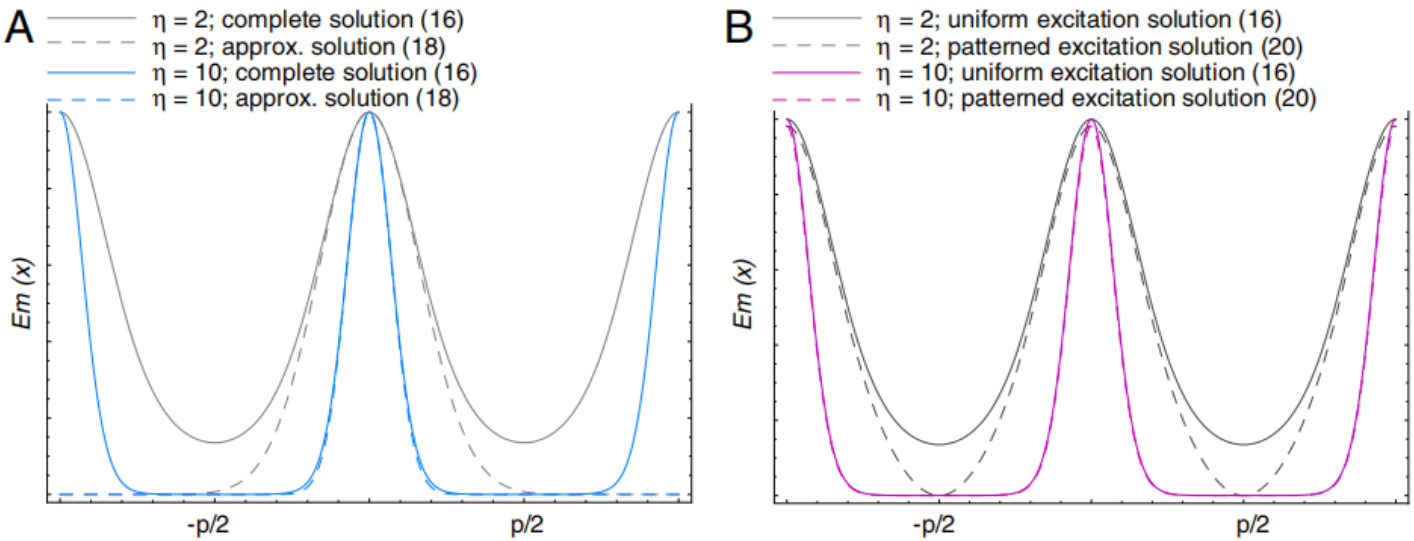
- **3': Expose by structured illumination**

$$I_{exp}(\mathbf{x}) = \frac{I_0}{2}(1 - \cos(2\pi k_0 \mathbf{x} + \phi + \pi))$$

Moreover, the photoswitchable molecule Dronpa we chose to use in our study is excited and driven to the off state with the same wavelength of light.While the fluorescence is being detected, the molecules will be driven to the dark state.Total expoosure time and saturate factor are T_2, η_2

$$\begin{aligned}
\mathrm{Em}(\mathbf{x}) &= \frac{1}{\tau_{\mathsf{off}}} \int_0^{T_2} I_{\mathsf{exp}}(\mathbf{x}) e^{\frac{-}{\tau_{\mathsf{offlig}}} I_{\mathsf{exp}}(\mathbf{x})} S(\mathbf{x}) dt \\
\mathrm{Em}(\mathbf{x}) &= \int_0^{\eta_2} I_{\mathsf{exp}}(\mathbf{x}) e^{-\eta[1-\cos(2\pi k_0 \mathbf{x} + (\varphi+\pi))]} S_{\mathsf{on}}(\mathbf{x}) d\eta.
\end{aligned}$$

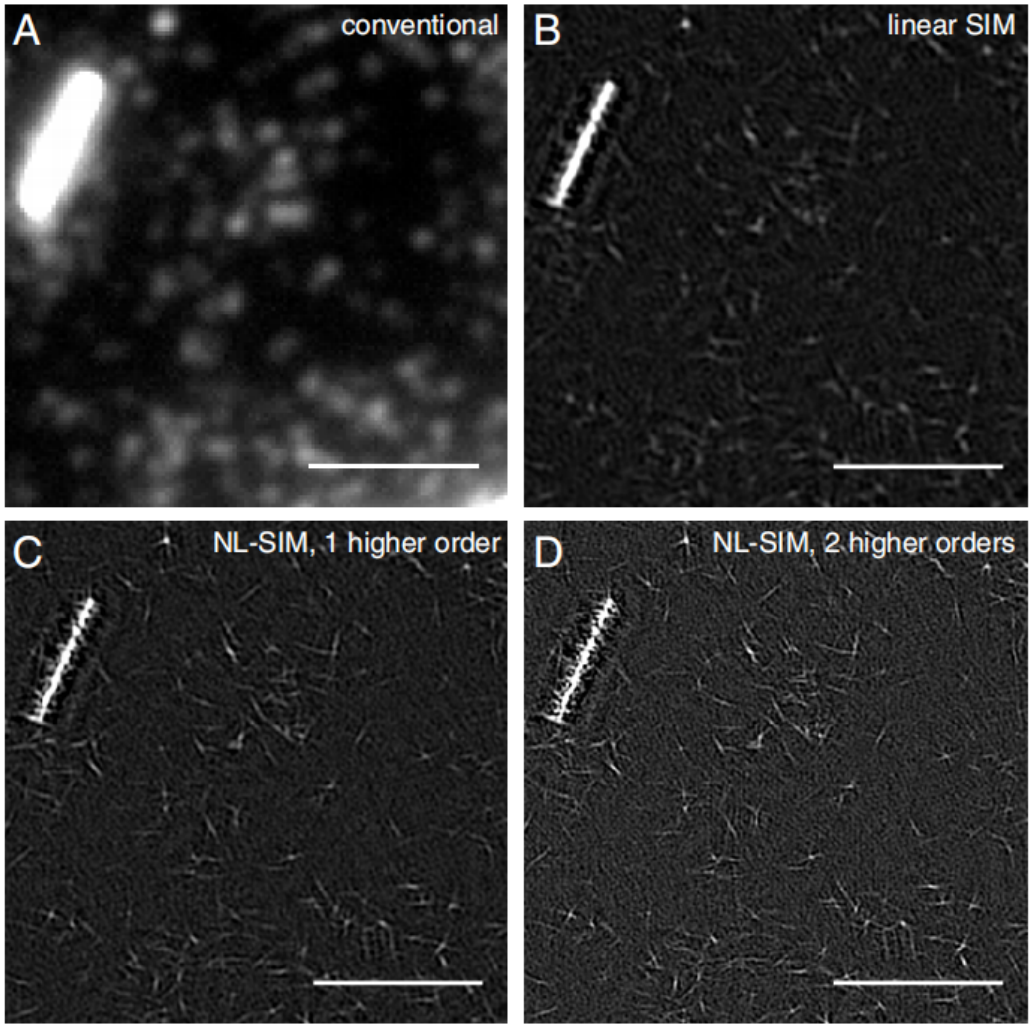
NL-SIM with Photoswitchable Moleculars



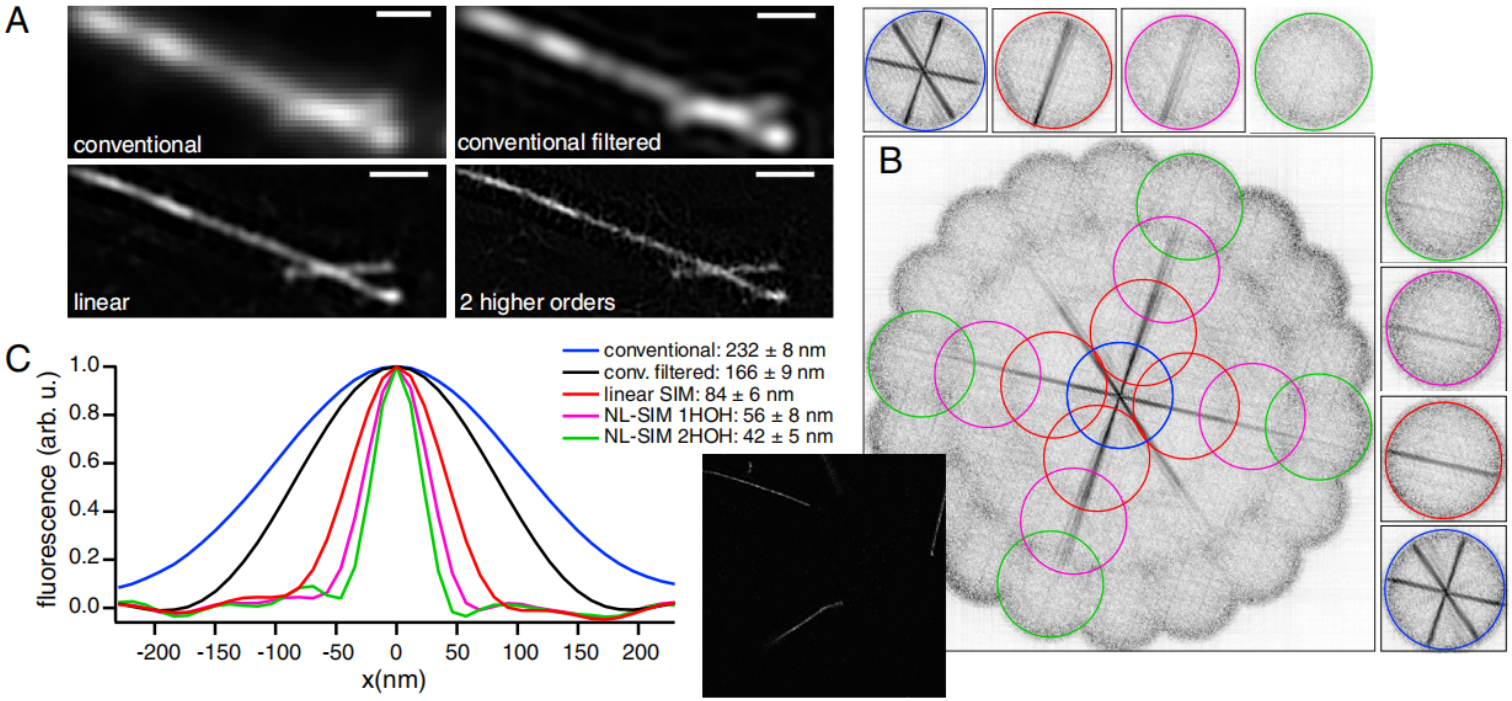
The effect of the patterned excitation (dashed) light can be seen at low saturation (gray) because the zero of the excitation light affects the nonzero trough of the patterned-on molecules (solid). At high saturation (pink), there is no effect of the patterned excitation light compared to uniform excitation

Results

The raw data is shown in movie. The comparison of different microscopy method is shown below



Results


A
conventional
conventional filtered
linear
2 higher orders
C
conventional: 232 ± 8 nm
conv. filtered: 166 ± 9 nm
linear SIM: 84 ± 6 nm
NL-SIM 1HOH: 56 ± 8 nm
NL-SIM 2HOH: 42 ± 5 nm
fluorescence (arb. u.)
1.0
0.8
0.6
0.4
0.2
0.0
-200 -150 -100 -50 0 50 100 150 200
x(nm)
B

Thanks! & Questions?

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