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Principle of single-molecule imaging

To capture single molecule signal, two conditions must be achieved. (ref)

> 1. High enough contrast
> 2. Only one ON-state molecule in the diffraction-limited region

AIM: High SBR and sparse

PALM:
High SBR & Sparse: bleaching

STORM:
High SBR & Sparse: OFF state dominant

The bleaching in PALM is time-consuming, especially in spinning disk.

For PALM, typical bleaching time takes about 0.5-1s, and frame number is about 10^4 to 10^5 frames. (ref) (2-10 hrs in total)

PALM video

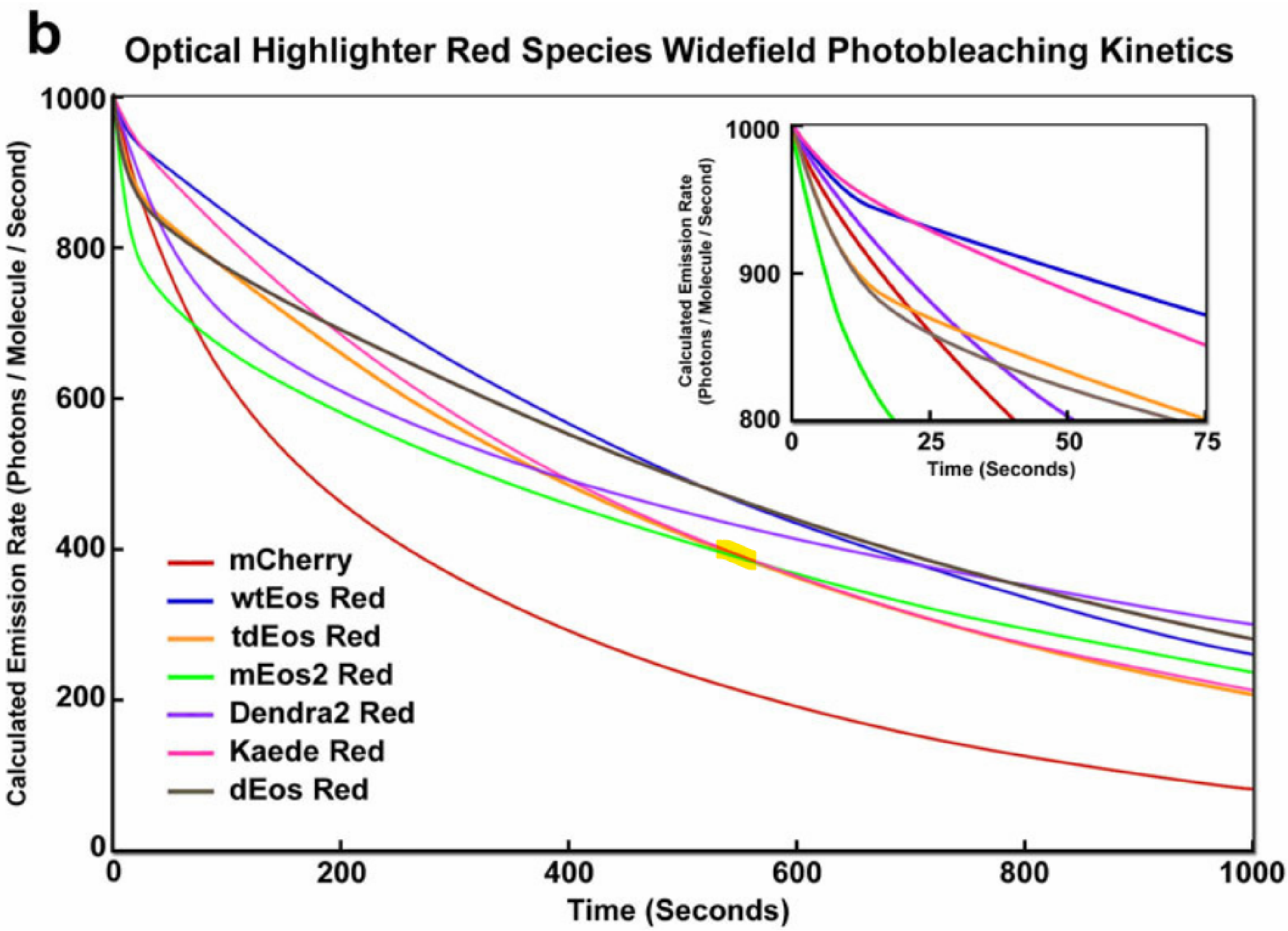
Comparison between widefield bleaching and confocal bleaching

Data ref

Condition:

1. Widefield: 40x dry objective (Nikon Plan Fluorite, NA = 0.85), metal halide illumination source
2. Confocal: 40x oil immersion objective (Olympus UPlan Apo, NA = 1.00), 543-nm laser
3. Laser intensity = ~475 mW/cm^2 power (Widefield), 100μW (Confocal)

Widefield



Confocal

> In most cases, the calculated $t_{1/2}$ for confocal bleaching is more than an order of magnitude greater than that for widefield illumination. (ref)

$t_{1/2}$: time to bleach 50% starting from 1000 photons/s emission per chromophore

Theory explanation

ref

Parameters (bleaching)

P: power
A: scanning area
N: number of beamlet
ω_1, ω_2: the radial, the distances from the center of the volume element in the radial and axial direction, respectively, at which the laser intensity has dropped by a factor of e^2, assuming a Gaussian beam profile.

> Photobleaching often takes place from the triplet state after intersystem crossing in the excited fluorophore. (Let's ignore k_2 for a while, i.e., $k_2 << 1$)
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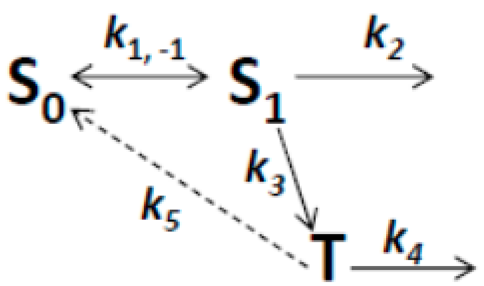
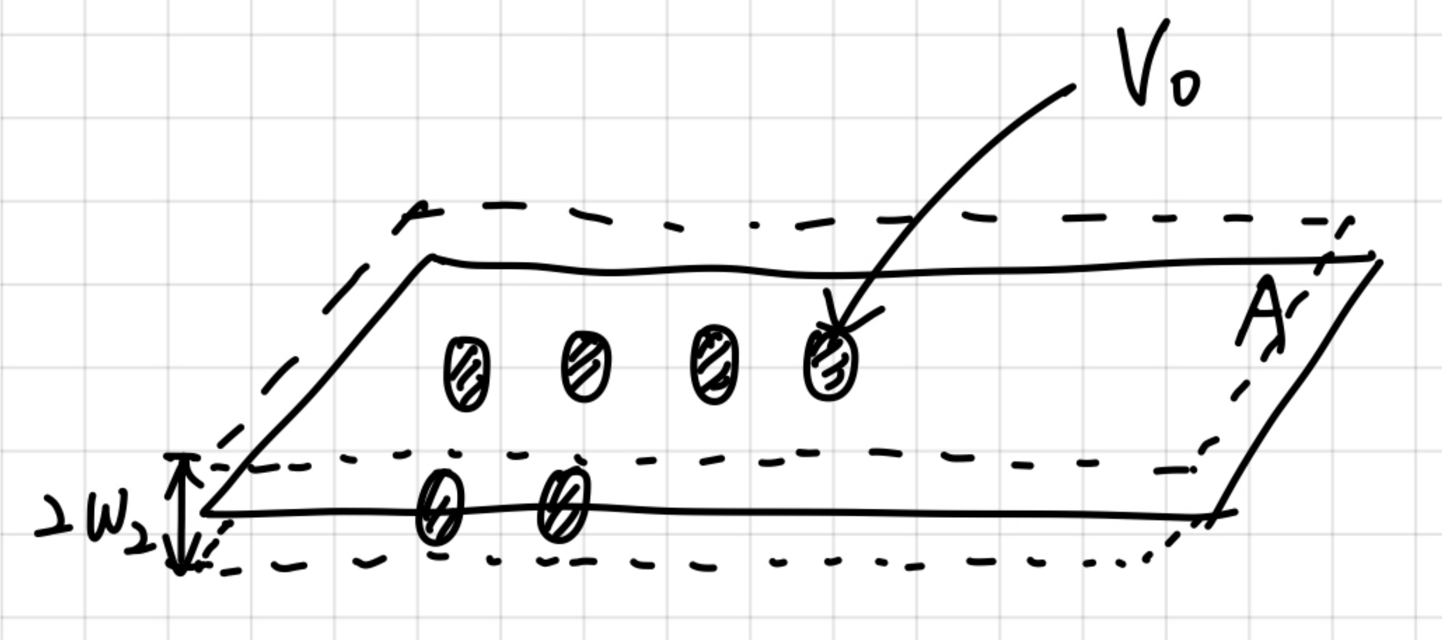
> 
>
>
> According to this model, triplet state population (in equilibrium) is important!

Plugin the parameters (bleaching)

$P = 20mW, A = 300\mu m * 300\mu m, N = 100, \omega_1 = 0.2\mu m, \omega_2 = 0.5\mu m, V_0 = \frac{4}{3}\pi\omega_1^2\omega_2 = 8.38 * 10^{-20}m^3$



Spinning disk

Consider the limiting case 1: the disk is not spinning.

1. At the excitation points:$P = 20mW \Rightarrow T_{eq,spot} \approx 0.63$
2. Outside the excitation points: $P = 0 \Rightarrow T_{eq,o} = 0$
3. $T_{eq} = T_{eq,spot} * \frac{N\pi\omega_1^2}{A} + T_{eq,o} * \frac{A-N\pi\omega_1^2}{A} = 0.63 * \frac{100*\pi*(0.2\mu m)^2}{300\mu m*300\mu m} = 1.39 * 10^{-4}$

Consider the limiting case 2: the disk spins very fast $\Rightarrow$ can be considered as a uniform widefield light source.

1. $P_{eff} = P * \frac{N\pi\omega_1^2}{A} = 2.8\mu W \Rightarrow T_{eq} \approx 0.0037$

Between two limiting cases, $T_{eq} \approx 1.39 * 10^{-4}$~$3.7 * 10^{-3}$

$r_{bleach,spin} = k_4 * 1.39 * 10^{-4}$~$k_4 * 3.7 * 10^{-3}$

Widefield

Widefield bleaching rate is the same as the second case of spinning disk. $\Rightarrow r_{bleach,wide} = k_4 * 3.7 * 10^{-3}$

> $\Rightarrow$ (Spinnig disk) confocal bleaching time is more than an order of magnitude greater than that for widefield illumination. **Agreed with observation!**

Why we need that many frames?

Nyquist condition. Can be calculated...

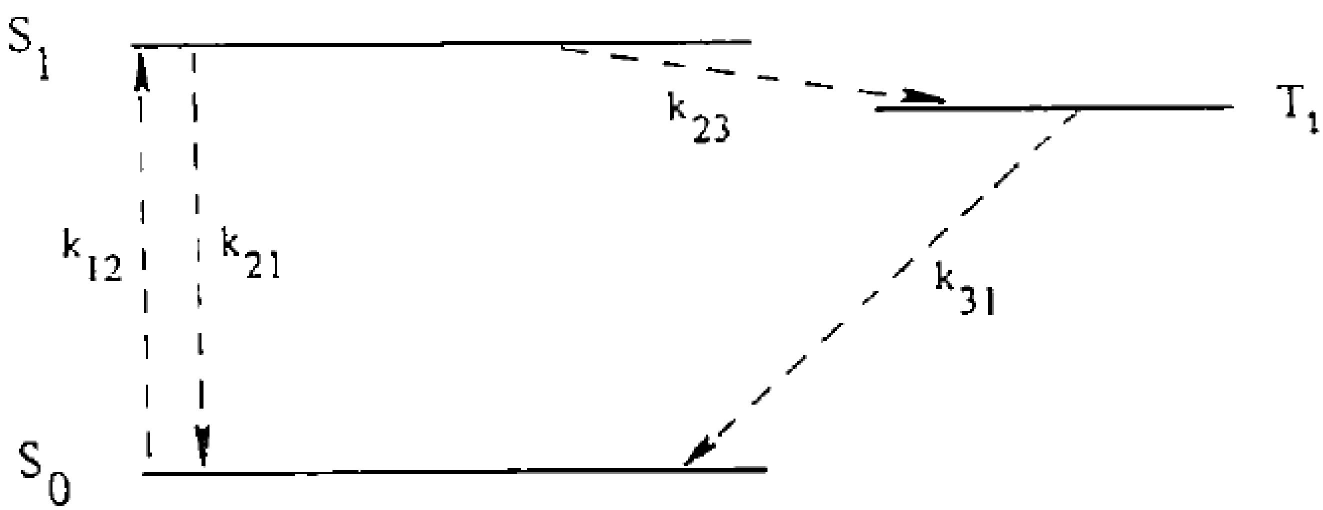
STORM is less time consuming.

> Unlike PALM that has fixed (ON-state) bleaching time, the ON/OFF states dynamics in STORM can be adjusted by external means (chemical condition, excitation power). $\Rightarrow$ **less time consuming.**

The photoswitching mechanism (Theory)

打561 Kaede會不會回到488的態? 也沒關係，還是在dark state

A three-state model. Using this model can explain why there is fast blinking.



Mathematically, we can use three ODE to describe the above system.
$\frac{d}{dt}\begin{pmatrix} S_0(t) \\ S_1(t) \\ T(t) \end{pmatrix} = \begin{pmatrix} -k_{12} & k_{21} & k_{31} \\ k_{12} & -(k_{23}+k_{21}) & 0 \\ 0 & k_{23} & -k_{31} \end{pmatrix} \begin{pmatrix} S_0(t) \\ S_1(t) \\ T(t) \end{pmatrix}$
Solve for the eigenvalue equation

$$\lambda(\lambda^2 + (k_{23}+k_{21}+k_{12}+k_{31})\lambda + k_{12}k_{23} + k_{31}k_{23} + k_{31}k_{21}) = 0$$

for the middle matrix. We have three eigenvalues $\lambda_1 = 0, \lambda_2, \lambda_3$, and corresponding eigenvectors $\mathbf{x_1}, \mathbf{x_2}, \mathbf{x_3}$

Hence, the general solution of $X(t) = \begin{pmatrix} S_0(t) \\ S_1(t) \\ T(t) \end{pmatrix} = c_1 e^{\lambda_1 t}\mathbf{x}_1 + c_2 e^{\lambda_2 t}\mathbf{x}_2 + c_3 e^{\lambda_3 t}\mathbf{x}_3$

- Since $\lambda_1 = 0$, the system will approach to a steady state when $t \to \infty$.
- λ_2 is a very large term. Hard to be measured.

- λ_3 can be measured. Assuming $k_{12}+k_{21} >> k_{31}+k_{23}$, then $\lambda_3 = -k_{31} - \frac{k_{12}k_{23}}{k_{12}+k_{21}}$, $T_{eq} = \frac{k_{23}k_{12}}{k_{12}(k_{23}+k_{31})+k_{31}(k_{21}+k_{23})}$

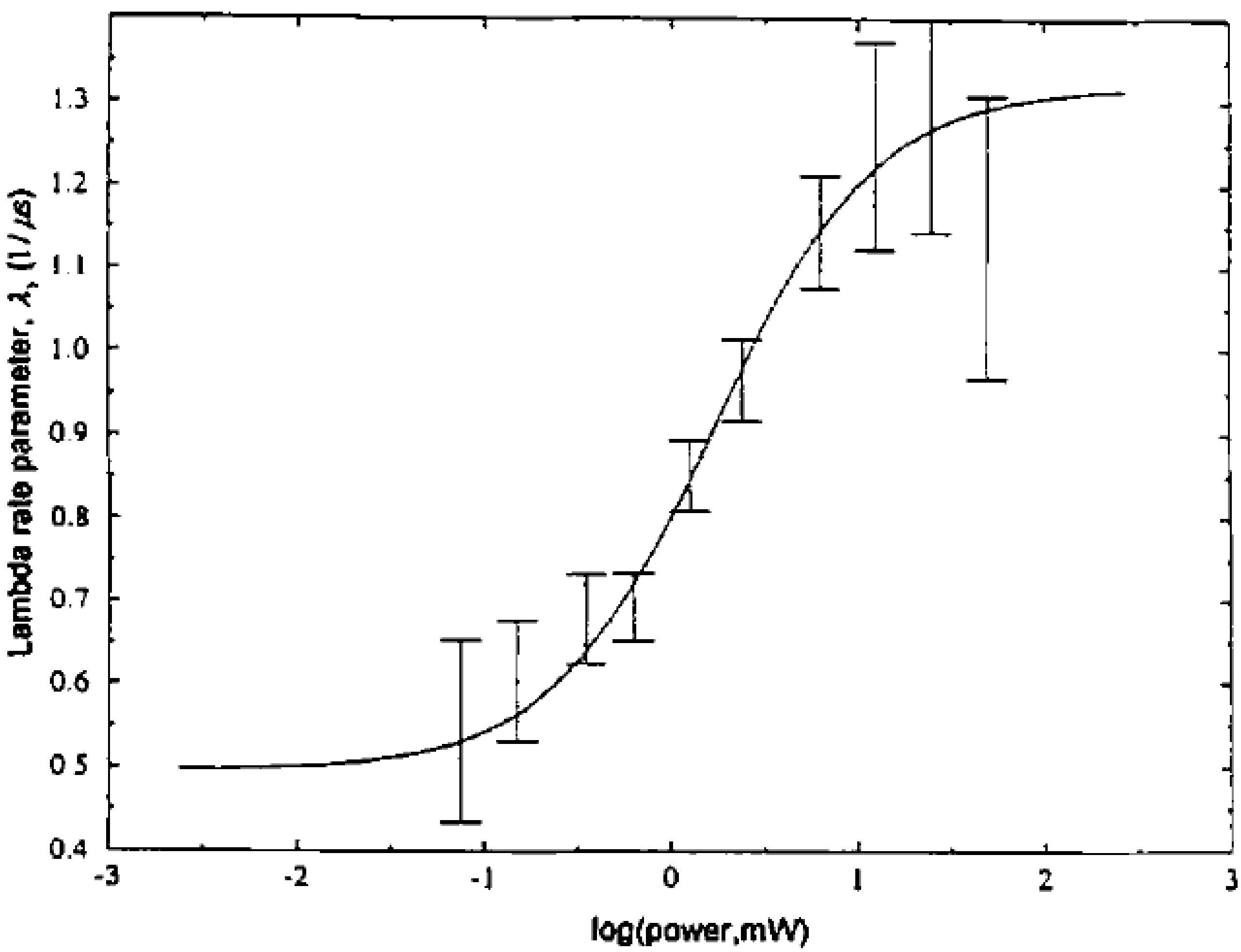
Combining the following effects to autocorrelation function:

- diffusion of molecule in and out the laser volume
- molecules entering and leaving triplet state

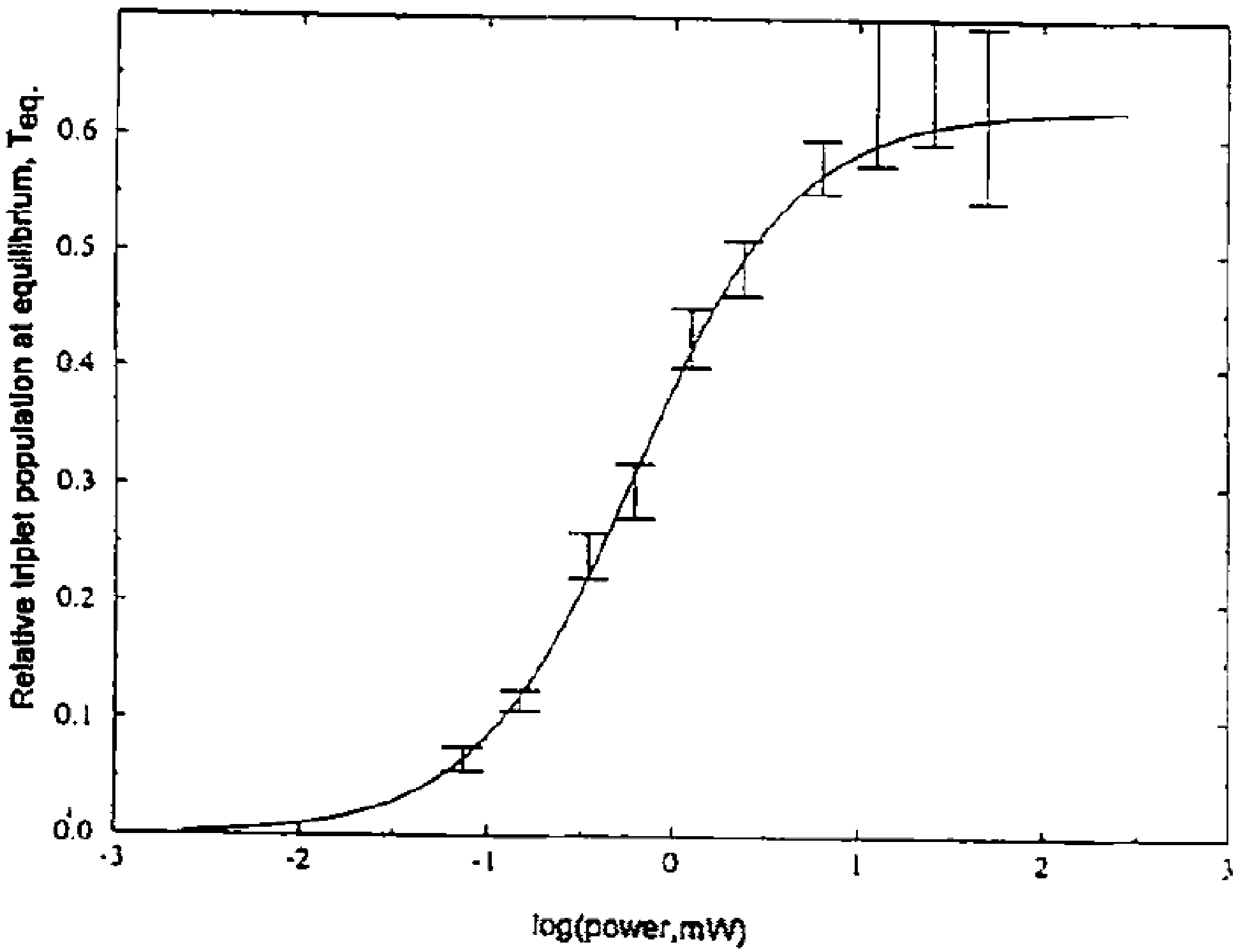
  We have our autocorrelation function

$$g(\tau) = 1 + \frac{1}{N}\left(\frac{1}{1+4D\tau/\omega_1^2}\right)\left(\frac{1}{1+4D\tau/\omega_2^2}\right)^{1/2} \times (1 - T_{eq} + T_{eq}e^{-\lambda_3\tau})$$

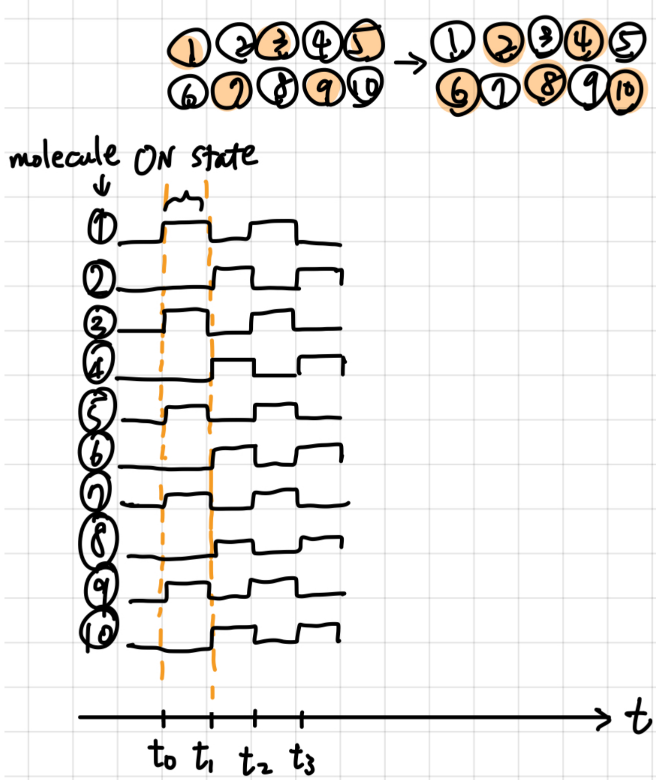
A typical relation between λ_3(the rate at which there is a population buildup in the triplet state) and intensity.



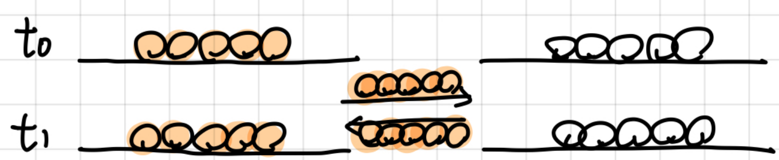
Relation between T_{eq} and intensity



Physical meaning of λ_3 and T_{eq}



$$\Rightarrow T_{eq} = 50\%$$



$$\lambda_3 = \frac{5}{t_1 - t_0} = \frac{1}{\frac{t_1 - t_0}{5}}$$

$\Rightarrow$ **Blinking rate** is dependent on λ_3 and labelling density ρ.

How to calculate blinking rate?

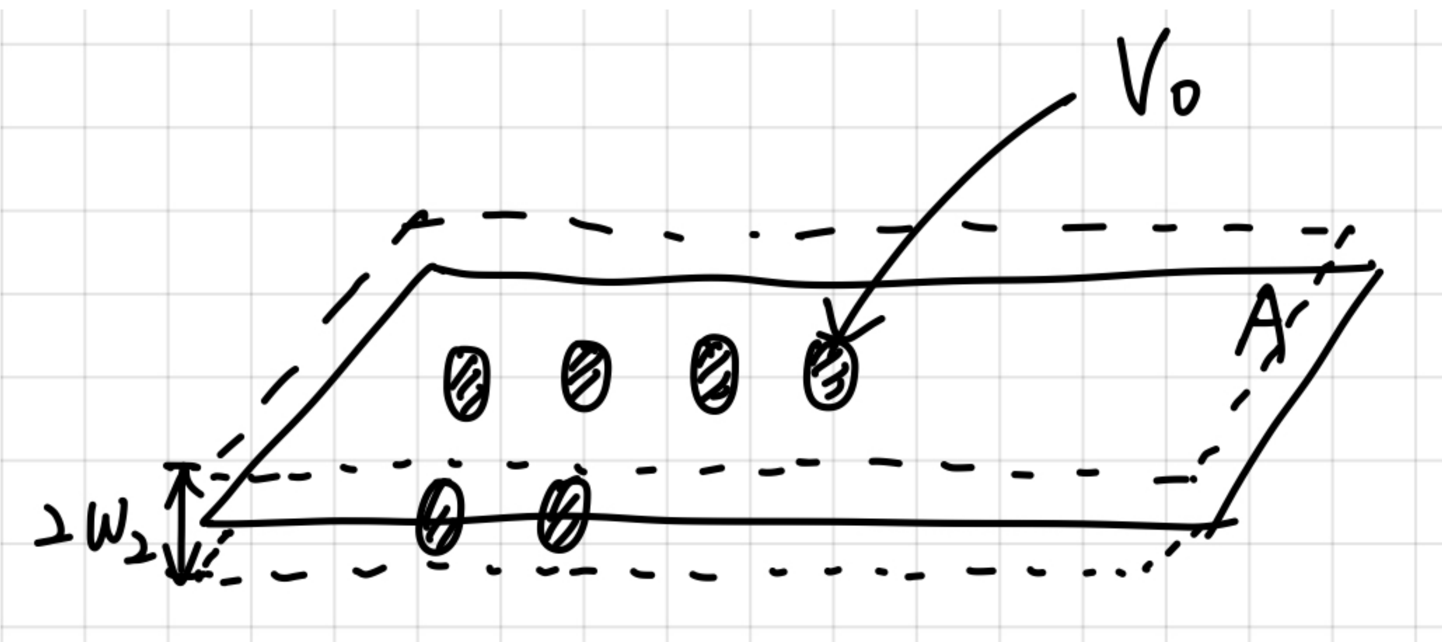
Parameters:

ρ: labeling density
N: number of beamlet
ω_1, ω_2: the radial, the distances from the center of the volume element in the radial and axial direction, respectively, at which the laser intensity has dropped by a factor of e^2, assuming a Gaussian beam profile.
$V_0 = \frac{4}{3}\pi\omega_1^2\omega_2$: volume of one excitation beamlet
$NV_0\rho = \frac{NV_0}{d^3}$: total number of molecules in the excitation volume.

P: power of total beamlets$\Rightarrow$ $\frac{P}{N}$: power of individual beamlets
A: scanning area
λ_3: the rate at which there is a population buildup in the triplet state
T_{eq}: relative triplet state population in equilibrium

Plugin the parameters

$$P = 20mW, A = 300\mu m * 300\mu m, N = 1000, \omega_1 = 0.2\mu m, \omega_2 = 0.5\mu m, V_0 = \frac{4}{3}\pi\omega_1^2\omega_2 = 8.38 * 10^{-20}m^3$$



Consider the limiting case 1: the disk is not spinning.

1. At the excitation points:$P = 20mW$ and split into 1000 beams $\Rightarrow T_{eq,spot} \approx 0.025, \lambda_{3,spot} = 0.5(1/\mu s)$

2. Outside the excitation points: $P = 0 \Rightarrow T_{eq,o} = 0, \lambda_{3,o} = 0.5(1/\mu s)$
3. $T_{eq} = T_{eq,spot} * \frac{N\pi\omega_1^2}{A} + T_{eq,o} * \frac{A-N\pi\omega_1^2}{A} = 0.025 * \frac{1000*\pi*(0.2\mu m)^2}{300\mu m*300\mu m} = 5.5 * 10^{-5}$
4. $\lambda_3 = \lambda_{3,spot} * \frac{N\pi\omega_1^2}{A} + \lambda_{3,o} * \frac{A-N\pi\omega_1^2}{A} \approx 0.5$

Consider the limiting case 2: the disk spins very fast $\Rightarrow$ can be considered as a uniform widefield light source.

1. $P_{eff} = P * \frac{N\pi\omega_1^2}{A} = 28\mu W \Rightarrow T_{eq} \approx 0.035, \lambda_3 \approx 0.5(1/\mu s)$

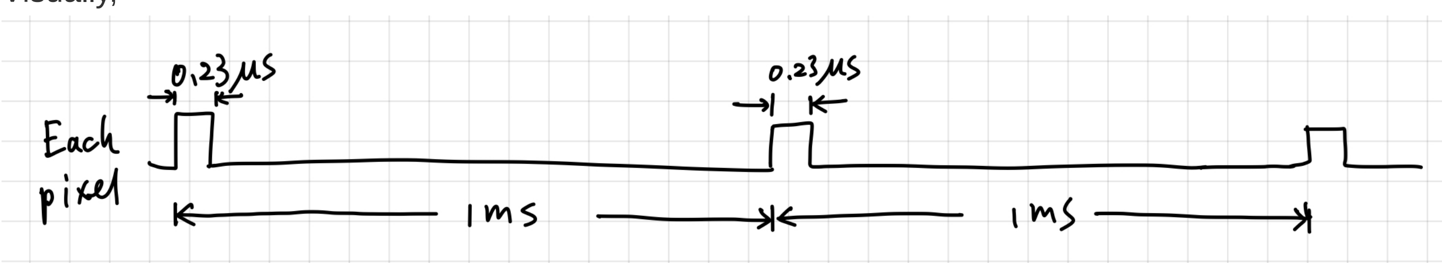
> Between two limiting cases, $T_{eq} \approx 5.5 * 10^{-5}$~$0.035, \lambda_3 \approx 0.5(1/\mu s)$

Case 3: pixel dwell time$\approx 0.1 \times \frac{1}{k_{23}} = 10^{-7}s = 0.1\mu s$

Case 4: pixel dwell time$\approx \frac{1}{k_{23}} = 10^{-6}s = 1\mu s$

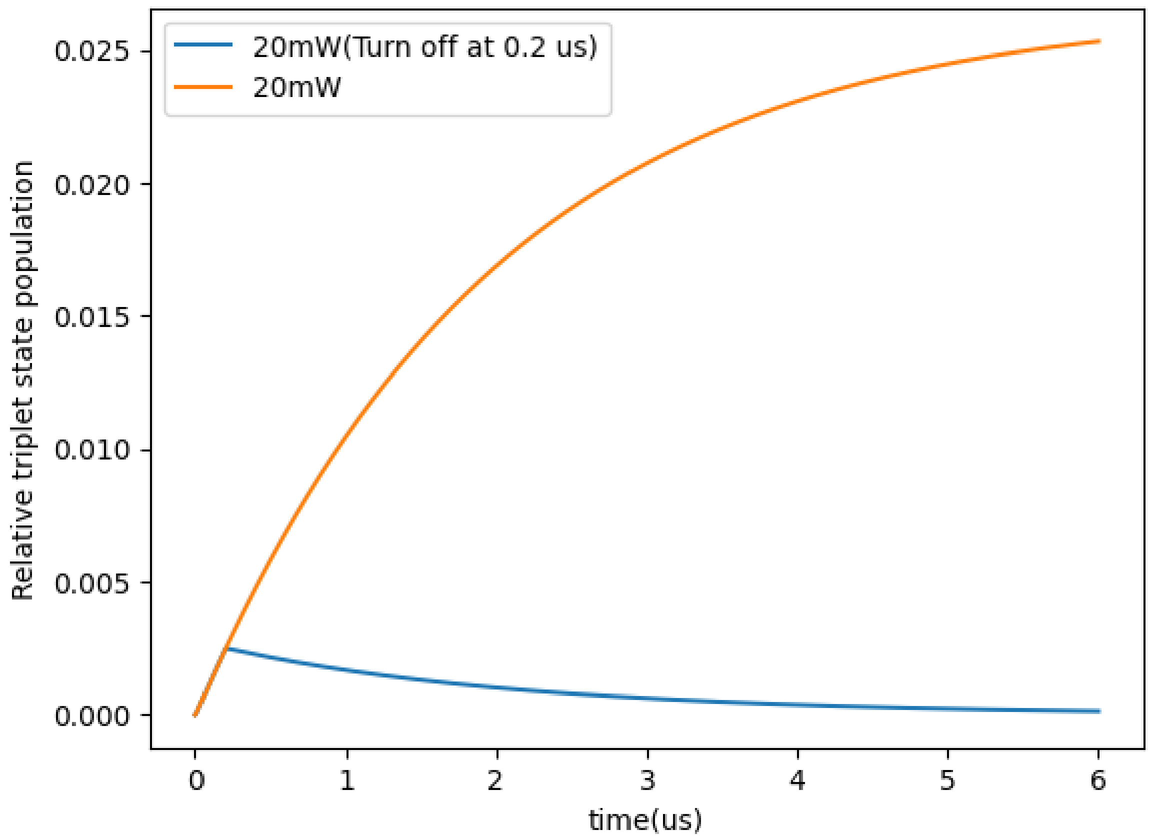
Case 5: pixel dwell time$\approx 10 \times \frac{1}{k_{23}} = 10^{-5}s = 10\mu s$

Current state of the art spinning disk rotates 15,000 rpm.ref1
The Nipkow disk is located in a conjugate image plane and a **partial rotation** of the disk scans the specimen with approximately 1000 individual light beams that can traverse the entire image plane in less than a millisecond.
ref2
Suppose that there are 2048 by 2048 pixels.
Then each beam passes $2048 * 2048/1000 = 4194pxls$ in 1 ms. Hence, pixel dwell time $\approx 0.23\mu s$ And each beam has power $20mW/1000 = 20\mu W$
Hence the intensity is $\frac{20\mu W}{\pi\omega_1^2} \approx 11kW/cm^2$ (Normally, widefield intenisty is about $2kW/cm^2$, an order smaller.)
Visually,



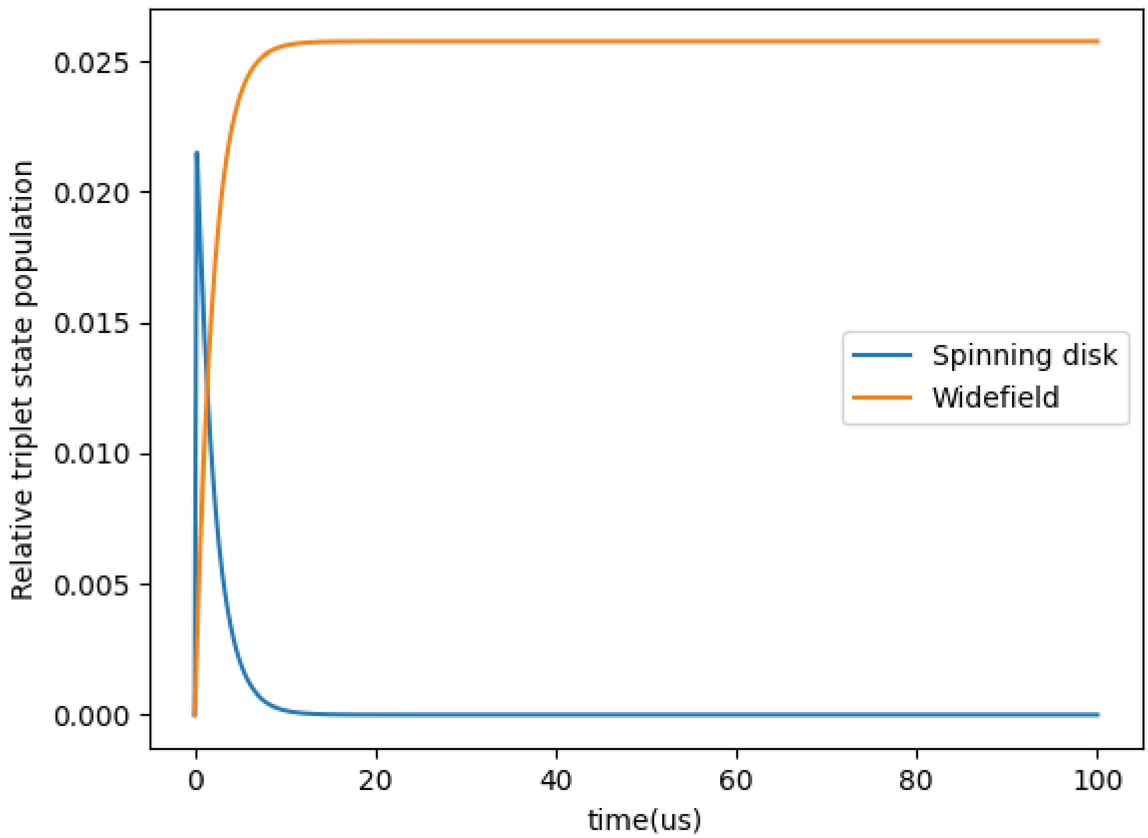
> Hence the state-of-the-art spinning disk can be thought as Case 3.
> Using the formula $X(t) = \begin{pmatrix} S_0(t) \\ S_1(t) \\ T(t) \end{pmatrix} = c_1 e^{\lambda_1 t}\mathbf{x}_1 + c_2 e^{\lambda_2 t}\mathbf{x}_2 + c_3 e^{\lambda_3 t}\mathbf{x}_3$

After calculation, $T(t)$ is as follows and reaches ≈ 0.0026 at $0.2\mu s$ and then decrease to 0 in $5\mu s$ ($P = 20mW$).



> Hence, the triplet development is not sufficient during this short time period.

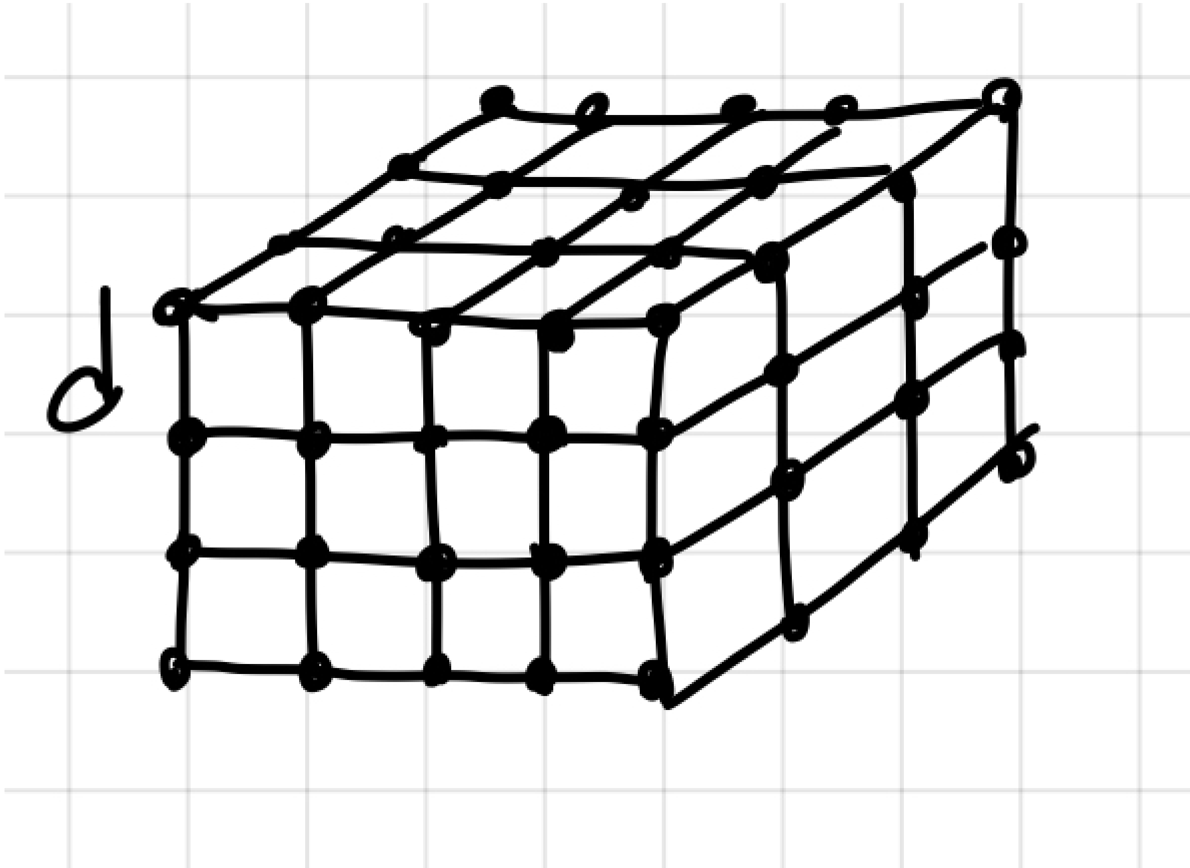
> **Final comparison**: spinning disk vs widefield, condition:
> $I_{spinning} = 11kW/cm^2$
> $I_{wide} = 1.1kW/cm^2$
> Spinning disk: $0.2\mu s$ pulse followed by $> 100\mu s$ rest per pixel
> Widefield: continuous illumination



Conclusion: Triplet state population is way more larger in the case of widefield than in spinning disk.

How to determine the labeling density?

Let's assume the labeling density ρ is $\frac{(L/d)^3}{L^3} = \frac{1}{d^3}$
$d = 1, 5, 10, 20, 50, 100nm$



Rh6G photophysical properties (Case study)

After several fitting procedures, we have

Table I. Obtained Values of the Rate Constants for Rh6G[a]

Excitation cross section (10^{-16} cm^2) (at 514 nm excitation wavelength)	1.3 ± 0.4 (1.7)
k_{21} (10^6 s^{-1}) (measured by pulsed laser and TCSPC)	250 ± 20 (200)
k_{23} (10^6 s^{-1})	0.9 ± 0.2 (0.4)
k_{31} (10^6 s^{-1})	0.5 ± 0.1 (0.3)

[a] Referenced values within parentheses.

We can also obtain $k_{12} = \sigma_{exc} \times \frac{P}{\pi\omega_1^2}$

$\frac{P}{\pi\omega_1^2}$ needs to be expressed as photons per square meter per second. $P = P_{origin} * 2.58 * 10^{18}$

DsRed photophysical properties (Case study)

Kaede photophysical properties (Case study)

Intensity dependence of ACF (The most important application)

Here is the experimental results of ACF curve of red form Kaede when varying the excitation intensity (from 2.5 to $78\frac{kW}{cm^2}$).

After fitting with the model

$$G_{\text{blink}}(\tau) = G(0)G_{\text{diff}}(\tau)\prod_{\text{i}} X_{\text{i}}(\tau)$$

$$X_{\text{i}}(\tau) = (1 - F_{\text{i}})^{-1}(1 - F_{\text{i}} + F_{\text{i}}\exp(-\tau/\tau_{\text{i}})),$$

, where τ_i is the rate, and F_i is the fraction, we have two blinking dynamics (i=2) with $\tau_1 = 6.5$ - 32 kHz(153μs-30μs), $F_1 \approx 30\%$, and $\tau_2 = 1.3$kHz - 10kHz(769μs-100μs), $F_2 \approx 20\%$.