Stimulated Emission Depletion (STED) Microscopy

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Aims of the practical

In this practical we discuss and demonstrate the principle of stimulated emission depletion microscopy (STED) [1]. This a super-resolution microscopy technique used to achieve resolutions down to 40 nm by adding a donut shaped "depletion beam" to a confocal laser scanning microscope to "switch-off" fluorescence outside the centre.

• Understanding of some of the applications of fluorescence microscopy and the advantages of super-resolution microscopy

• Understanding the principle of STED, where the resolution enhancement comes from and what limits the resolution. Show how resolution improves with increasing laser power

• Demonstrate some of the design considerations in building a STED microscope including:

  The use of pulsed lasers

  Supercontinuum sources

  Spatial light modulators for aberration correction

Questions

- How can the theoretical limit of diffraction be surpassed? Can clever tricks be devised to get over this limit?
- What is the physical principle behind a STED microscope?
- What are the advantages of supercontinuum generation in a STED microscope?
- How can a donut beam be generated? What are the considerations important in generating this beam?
- What are the spatial and temporal constraints of the laser beams in STED?

Background

Fluorescence is a luminescent process and occurs when a fluorescent molecule, often referred to as a fluorophore, is transferred to an excited state by the absorption of a photon and returns to a lower energy state by the emission of a photon. The phenomenon of fluorescence is best described using Jablonski diagrams [2]. A simplified example is shown in figure 1. This represents a typical fluorescence process where a molecule in the ground state is transferred to the first excited state ($S_0 \rightarrow S_1$) by the absorption of a photon of energy $h\nu_{abs}$. The fluorophore then relaxes to the ground state ($S_1 \rightarrow S_0$) by emitting another photon of energy $h\nu_{em}$.
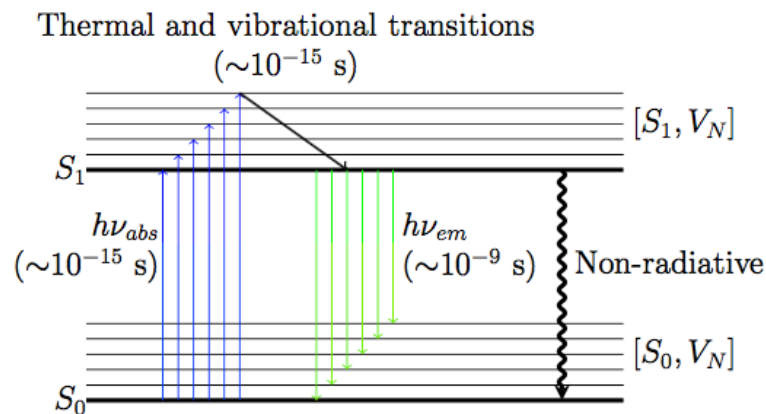
Figure 1: Simplified Jablonski diagram of fluorescence. Molecules are excited from the ground state (S_0) to an excited state ($[S_1, V_N]$) through the absorption of a photon of energy $h\nu_{abs}$. Thermal and vibrational transitions transfer the molecule to the lowest energy level of the excited state (S_1). The molecule then transfers to the lower electronic energy level ($[S_0, V_N]$). This process releases a photon of energy $h\nu_{em}$. S_0 and S_1 represent the ground and and first excited singlet states respectively. V_n represent the vibrational energy states associated with S_0 and S_1. A molecule in the excited state can also decay by a non-radiative transition. The duration of each process is shown in brackets.

Because the wavelengths emitted are spectrally distinct from the wave- length used to excite the molecule the excitation light can be filtered in detection. This makes fluorescence detection a low noise, high SNR, technique.

Fluorescent molecules can be conjugated to biological molecules either by genetic expression, anti-body conjugation or direct labelling. This allows the specific sensing of the labelled molecules in a sample.

Discussion: discuss briefly how fluorescence is used in sensing applications and the advantages of different labelling approaches. Fluorescence microscopes – such as confocal microscopes – can be thought of as spatially resolved fluorescence sensors, locating fluorescent molecules bound to specific chemical structures.

Confocal Microscopy

In confocal microscopy the sample is imaged using a single excitation point formed in the sample by focusing the excitation source in the sample plane using a microscope objective. Fluorescence emission is also collected by the objective. Although fluorescence is still excited outside of the focal plane a pinhole is used to reject out of focus fluorescence. The pinhole acts as a spatial filter and will accept only light originating from the focal plane. As shown in figure 2.
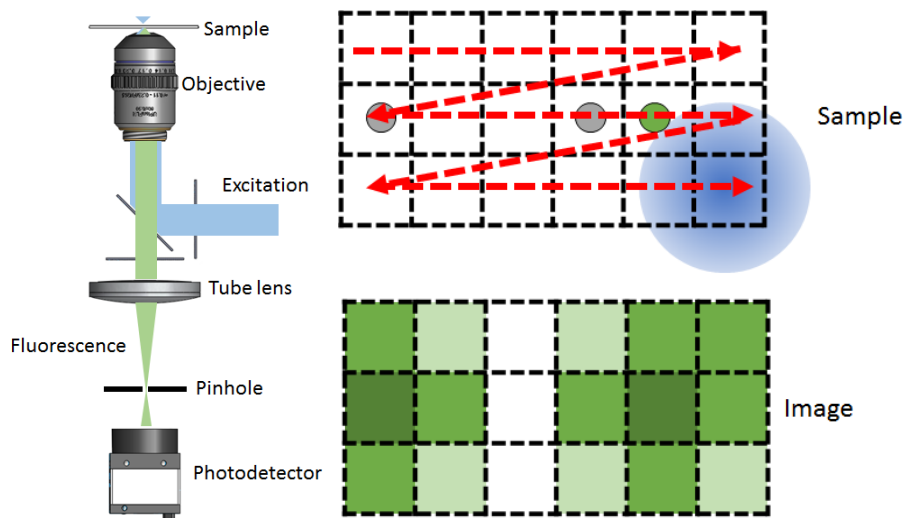

Figure 2: Schematic of a confocal microscope. The excitation light is focused on the sample and the fluorescence intensity measured. The fluorescence is focused onto a pinhole which is used to reject out of focus light. The sample is scanned pixel by pixel either my moving the sample or scanning the beam to form an image. The sample is imaged by scanning the excitation point through the sample - either through moving the sample or translating the beam using laser scanning mirrors. By translating the excitation point in three dimensions 3D reconstructions of the sample are possible with high signal to noise.

Resolution Limit

In confocal microscopy excitation light is focused on the sample. The fluorescence emission is assumed to have originated from this spot. Because light is a wave a lens acts as a diffractive element. The focal

spot is therefore a diffraction pattern defined by an Airy disc with radius defined by the wavelength of light λ and the numerical aperture NA.

A confocal image is the convolution of the focal spot with the sample. The image of a point object is referred to as the point spread function.

The spatial resolution of an imaging system is typically defined as the minimum distance between two point-like objects at which they can still be identified as distinct features (Δx_{min}) [3]. This is limited by the size of the point spread function. Figure 3 shows the point spread functions of two point objects with separations greater than, equal to and less than the diffraction limit.

● Emitters   ···· Emitter intensity   — Total intesnity

(a) $\Delta x > \Delta x_{min}$ (b) $\Delta x = \Delta x_{min}$ (c) $\Delta x < \Delta x_{min}$
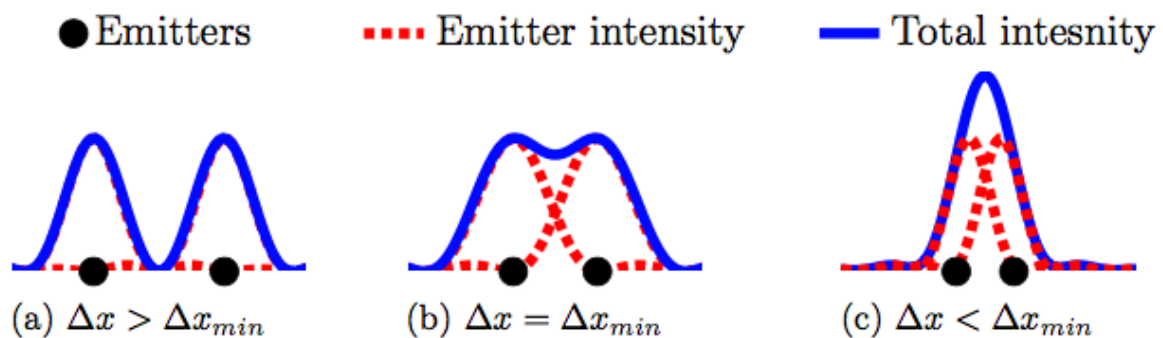
Figure 3: A one-dimensional demonstration of the diffraction limit. Point emitters are imaged as an Airy disk with FWHM given by the diffraction limit. Δx_{min} is the minimum separation between two features which can be resolved by an imaging system. Examples of a resolved (3a) a diffraction limited (3b) and an unresolved system (3c) are shown.

A simple definition of the diffraction limit is the Rayleigh limit. This is the distance from the centre of the PSF to the first minimum. This imposes a limit laterally of

$$\Delta x_{min}, \Delta y_{min} = \frac{\lambda}{2n \sin(\alpha)} = \frac{\lambda}{2\,NA} \qquad (1)$$

where λ is the wavelength of light and nsin(α) is the numerical aperture (NA).

For a fluorescence imaging system with λ=500 nm and and oil immersion objective with NA=1.4 the resolution limit would be

$$\Delta x_{min}, \Delta y_{min} \simeq 200 \text{ nm,} \tag{2}$$

This is much larger than biological molecules and sub-cellular structures. In order to study these structures it is therefore necessary to consider super- resolution microscopy.

STED microscopy

In confocal microscopy samples are typically raster scanned with a diffraction limited focal spot at the excitation wavelength (λ_{exc}). In order to enhance the resolution it is necessary to reduce the size of the effective focal spot. STED microscopies achieve this by overlaying the excitation laser spot with a spatially shaped beam which has a zero co-aligned with the centre of the beam. This is illustrated schematically in figure 4.

(a) Diffraction limited excitation spot

(b) Depletion beam transfers molecules to dark state
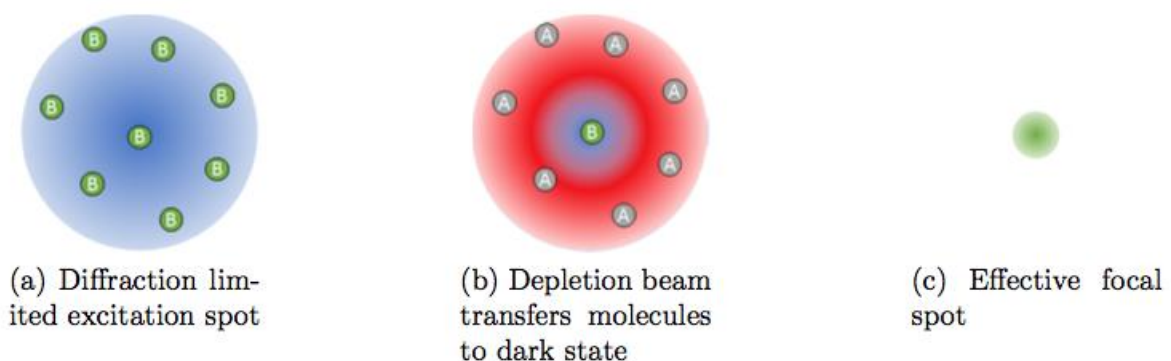
(c) Effective focal spot

Figure 4: Principle of STED and RESOLFT type microscopies. STED and RESOLFT are point scanning techniques, similar to confocal microscopy. Instead of scanning with a diffraction limited excitation focal spot the beam is overlaid with a depletion laser (λ_{depl} - shown

in red). This beam drives a depletion transition - which is distinct from fluorescence. Therefore fluorescence can be assumed to originate from the centre of the focal spot - giving a smaller effective focal spot size.

The second laser beam is at wavelength, λ_{depl}, which will deplete fluorescence by forcing fluorescent molecules in the bright state (B) into a, temporary, dark state (A). Therefore only fluorophores at the intensity zero can be in the bright state (B) so fluorescence emission can only originate from this reduced spot. The intensity of this laser can be raised such that it saturates the dark state everywhere outside of the intensity zero. This technique is therefore non-linear.

Systems for STED microscopy

The STED microscope used here is described by figure 9. In this practical we discuss and demonstrate the supercontinuum generation by a photonic crystal fibre (PCF) and aberration correction and donut beam generation using a spatial light modulator (SLM).
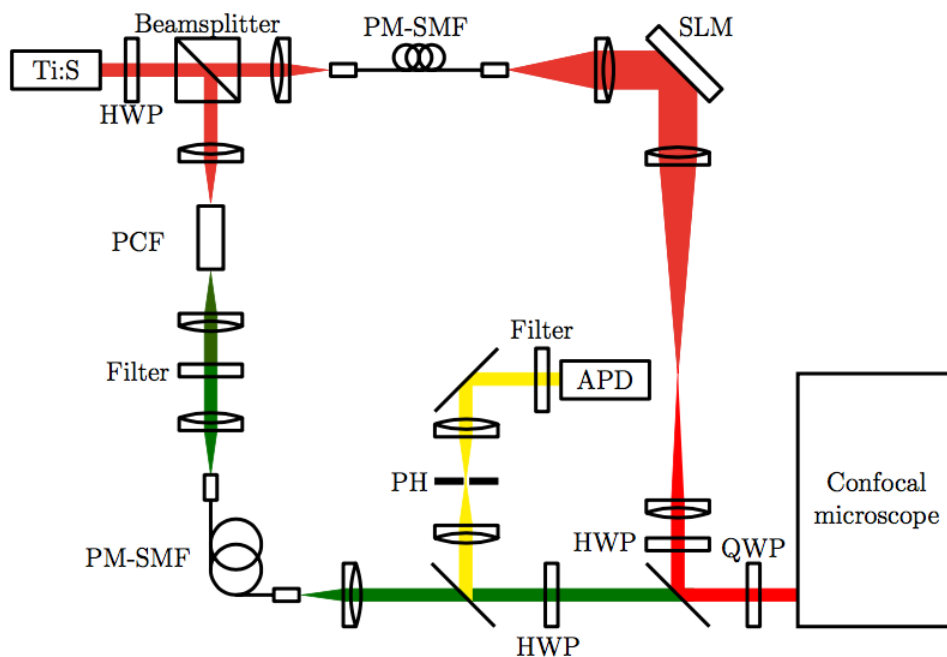

Figure 9: Simplified schematic of STED system. A titanium sapphire pulsed laser is split at a polarising beamsplitter to form both the

excitation and depletion beams, a half wave plate (HWP) is used to control how much power is used for each. The excitation beam is generated using a photonic crystal fibre (PCF) to generate a supercontinuum an excitation line at 640 nm is selected using a bandpass filter. The depletion beam is sent to a spatial light modulator (SLM) which is used to correct for aberrations and generate a donut shaped beam in the image plane. Both beams are recombined and sent to the microscope. Emitted fluorescence is selected by a dichroic mirror and filter and detected on an avalanche photodiode (APD).

Supercontinuum generation

A photonic crystal fibre is used to generate a supercontinuum. In this fibre the pulsed laser propagates through a non-linear medium at high intensities. A range of non-linear processes occur generating new wavelength. One of these is selected using a bandpass filter

Discussion Discuss the advantages in generating the excitation beam in this way.

Spatial Light Modulator

A spatial light modulator is used to generate the donut shape of the depletion beam by applying a helical phase mask. This element can also be used to coalign the depletion beams and excitation beams as well as to correct for aberrations

Demonstration Show a linear phase mask on the software, demonstrate how changing this between Gaussian and helical changes the beam profile on gold nanoparticles.

Demonstration Show how linear phase can be used to move the beam using gold nanoparticles.

Demonstration Show the difference between an aberration corrected donut and uncorrected.

Discussion Discuss practical advantages of SLM over physical optical

elements for STED

Depletion power and resolution

We derived a function (equation) for the resolution as a function of depletion beam power (equation 6). We can verify this relationship applies by measuring the PSF for a range of depletion powers on a point like object.

Practical work Image 20 nm beads with increasing depletion powers (moving to different field of view each time). Measure the FWHM of beads using ImageJ and fit to equation to measure I_{sat},

Discussion From the above we see that increasing the depletion power improves the resolution. What may be the limiting factors of this? On living samples? Fixed samples?

Applications of STED

Discussion In the context of other super-resolution microscopy techniques, what would the advantages of STED be?

Safe use of lasers in this practical

1. The Ti:Sa laser used in the STED microscope is a Class 4 laser. Class 4 lasers pose an eye hazard both if the beam is viewed directly and if a diffuse reflection of the beam is viewed (e.g. if the beam reflects from a piece of white paper). Hence Class 4 beams must be fully enclosed when they are used for microscopy, by having a light-proof illumination enclosure and also by covering the sample during imaging. Ti:Sa lasers are particularly hazardous for because the beam is infrared light and cannot be seen, hence users have no aversion response to the bright light, and need to use IR visualisation tools to even see the beam position.

2. Laser beams should be fully enclosed during normal use, to prevent any chance of direct exposure to laser light.
3. The light source enclosure must be closed during the actual measurement.
4. The microscopy specimens must be covered with a light-proof black box before the illumination shutter is opened.
5. The illumination part of this system must not be opened or aligned by students on this practical (it may only be aligned by the demonstrator). If this enclosure is open, then the following safety measures must be taken. (1) Thorlab LG9 (orange) laser goggles must be worn by all users in the STED bay if the Ti:Sa beam enclosure is open for alignment.
6. The Thorlabs LG9 goggles must also be worn by all users in the STED bay during imaging. Usually, the sample will be covered by a light-proof box, but goggles must also be provided to all people in the bay during STED imaging, in case the sample is briefly uncovered.

Further reading prior to the practical (required reading in bold)

- **[1] S W Hell and J Wichmann. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. Optics Letters, 19(11):780, June 1994.**

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