STED and GSDIM: Diffraction Unlimited Resolution for all Types of Fluorescence Imaging

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This article gives an overview of two different types of superresolution techniques, stimulated emission depletion (STED) microscopy and ground state depletion imaging (GSDIM). The two methods use very different approaches to reach the same goal: to see more details in light microscopes than possible when diffraction limited.

Keywords resolution, superresolution, STED, GSDIM, emission depletion, triplet states, diffraction limit

1. Diffraction Limitation

As was pointed out by Ernst Abbe, the limit of resolution in an optical microscope is ruled by the aperture of the objective and the color of the light(19) His reasoning was based on the minimum distance d between two objects at which they can still be individually distinguished. The simple relation he found stated the distance to be proportional to the wavelength and inversely proportional to the sine of the half opening angle. Later derivations, e.g. by integration of diffraction patterns and applying the Rayleigh criterion yielded similar results. It might be worth noting that all descriptions of resolution imply an arbitrary definition of what "resolved" actually means. Therefore, one should keep in mind that all numbers given for resolution do not mean that there is a sharp edge beyond which all of a sudden nothing is visible. And it is wise not to overemphasize the numbers that are discussed in this context, especially if they show more than 2 significant digits. Nevertheless, all definitions are good for comparison of the various methods, instruments and preparations. A well-established measure is the full-width half maximum of the intensity distribution of a structure that is assumed to be sufficiently smaller than the expected resolution. This is a very simple and quick measurement.

With Abbe's formula, we can estimate a rough resolution limit for standard light microscopy. Assuming light of 550nm and a numerical aperture of 1.4, the resolution $d=\lambda/2NA$ should reach ca. 200nm. If one wants to increase the resolution, a shorter wavelength or a higher NA is required. Higher NA is very costly and requires immersion and preparation media at a higher refractive index than that of glass or oil. Although many attempts have been made in that direction, no practicable and sensible method was figured out. Some good results were made with UV microscopes, where the resolution might reach 100nm. This is possible with light below 300nm, which again requires special and costly equipment, as there are not many glass types that transmit below 350nm. Besides the equipment, shorter wavelength increases scattering and is readily absorbed by many organic compounds. For similar reasons, but even harder by orders of magnitude, microscopy with x-rays did not find its way into laboratories.

In contrast, the very elaborate and costly electron microscopy(2) did find many fields of application due to the fact that the improvement in resolution as compared to light microscopy is not just a factor of 2 (UV microscopy) or 10 (x-ray microscopy), but reaches a factor of more than 1000 (sub-nanometer).

An entirely different approach to see smaller things than by diffraction-limited methods is near-field scanning optical microscopy. Here, a tiny glass tip is scanned over (or through) the sample. The resolution is defined by the size of the tip rather than by diffraction phenomena, enabling details down to some 30nm to be imaged. The obvious disadvantage of this method is the limited depth resolution. To obtain images, the tip has to be brought into the close vicinity of the object. Therefore, it is mostly a method for surface imaging of flat (sliced) objects.

Incidentally: it is of course possible to see objects that are much smaller than the resolution limit – given they are dispersed enough to locate them as individual objects. R. Zsigmondy(3) earned the 1925 Nobel Prize in chemistry for his work on the chemistry of colloids. He used a microscope where the illumination was tilted by 90°. When looking through the microscope into a cuvette containing colloidal gold particles in low concentration, he could see bright spots – like stars in the sky. The size of the spots was that of the diffraction pattern (like in astronomy), even if the objects were much smaller. The illumination from an angle is still known as dark field-microscopy today.

2. Stimulated Emission Depletion Microscopy

It is obvious that no microscope can resolve better than the diffraction limit, if the method for imaging is diffraction. This means there is no chance of improving the resolution just by focusing. The first approach to still see more details with ordinary lenses was proposed by S. Hell(4), who suggested using properties of the sample (here: the

fluorochromes) to obtain diffraction-unlimited resolution. Meanwhile, STED microscopes are commercially available and have a significant impact on understanding structure and function of biological objects beyond the diffraction limit.

2.1 Interaction of light and matter

When photons interact with matter (resonantly), three different transitions are possible. The first transition occurs when an incident photon has the appropriate energy (color) to fit to an available transition of the electron system of the matter. That matter may consist of atoms, molecules or large arrays of e.g. metals or semiconductors, where the electronic system is not localized to a single molecule. In any case, it is always the electrons that interact with the photons. If the electron system allows a higher energetic state, the difference to the current state being the energy of the photon, there is a probability of the photon energy being converted into the higher electronic state. In that case the photon disappears (is absorbed) and the molecule undergoes a transition into an excited state. We know this phenomenon from many daily experiences, currently relevant for example in solar thermal collectors (or in sunburn).

Once excited into a state of higher energy, the molecule has a certain probability to spontaneously release that energy which is converted into a photon and emitted. It is of no importance how the excited state was reached. This could be a result of absorption (see above) or other pump mechanisms. A glowing hotplate emits photons upon excitation with thermal energy. If the excitation was light (photons), the process is called fluorescence (simplest case). The energy of the fluorescence photon is usually less than the energy of the exciting photon due to fast transitions in thermal sub-states. That change in colour is called Stokes shift. The process is called spontaneous emission.

The last type of interaction was first theoretically predicted and is not an obvious everyday phenomenon. Once an electron system has assumed an excited state, the return into a lower state can be triggered by interaction with a photon that has an energy which fits to a possible transition from the excited into one of the thermal ground states. This process is called stimulated emission (as opposed to spontaneous emission). The emitted photon will have the same properties as the trigger photon: identical wavelength (colour), polarization direction, phase and direction of propagation. If one can excite many molecules in a medium into an excited state, an appropriate photon interacting with one of these molecules can cause two photons with identical parameters. These two can subsequently stimulate two more molecules to emit, yielding 4 photons. It is possible to repeat that process many times, with the result of a very intense and bright coherent light beam. This is the principle of a laser as it was first built by T. Maiman(5) in 1960. As mentioned above, the phenomenon of stimulated emissions was predicted beforehand. It was first discussed by A. Einstein(6) in 1916. Due to the rapid development of laser technology and its miscellaneous applications, stimulated emission has since become very much of an everyday phenomenon.

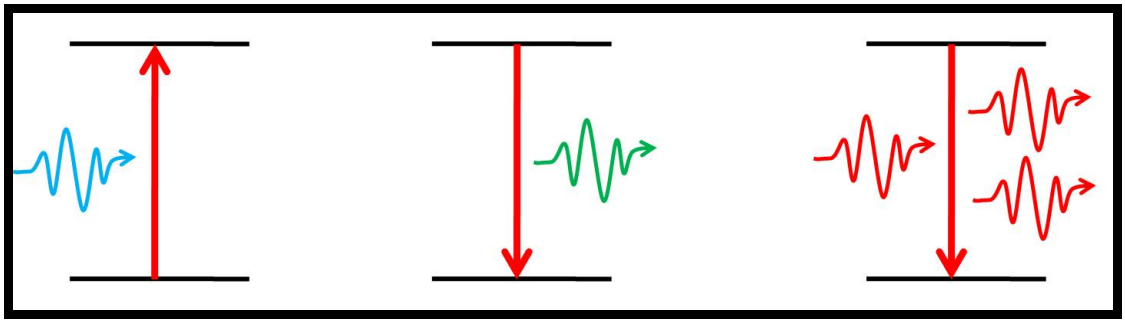

Fig. 1 The three modes of resonant interaction of light with matter: Spontaneous absorption, spontaneous emission and stimulated emission. For a detailed description: see text.

STED microscopy employs all these three different modes. It is a method that is based on fluorescence and increases the resolution by specifically applying stimulated emission.

2.2 The point spread function limits the resolution

When light is focussed by lenses, the focus cannot assume an infinitely small diameter. Even if the source is an infinitely small point (which is purely theoretical for an emitter of photons), there is no way to create an equally infinitely small image of that point by means of ordinary glass lenses (nor by extraordinary lenses). Rather, the lens geometry and size determines the kind of pattern representing the point in an image. A given lens will modify a given ray of light and create a modified ray of light that can be described by the “point spread function”. This function is the rule that describes how the point source is transferred into a pattern. The point is transferred into a pattern in the corresponding focus, but also all other layers parallel to the focal plane will feature different patterns which are determined by the rule. In general, the point spread function is therefore three-dimensional. For most purposes, also in our context, a treatment of the focal point is sufficient.

As optical instruments usually use circular tubes and lenses, the diffraction pattern of a spot generated in a microscope is a circular pattern. It is possible to calculate that pattern, if the numerical aperture and the wavelength are given. These two parameters are sufficient. The pattern is based on interference of the rays emerging from all points of the aperture, which in the simplest case is the area of the lens. This pattern is called "Airy pattern"(7) in honour of George Biddell Airy, who contributed much to such phenomena as an astronomer. It is this pattern that prevents optical systems from having infinite resolution: if two point-like structures sit within the inner disc, there is no way of telling them apart.

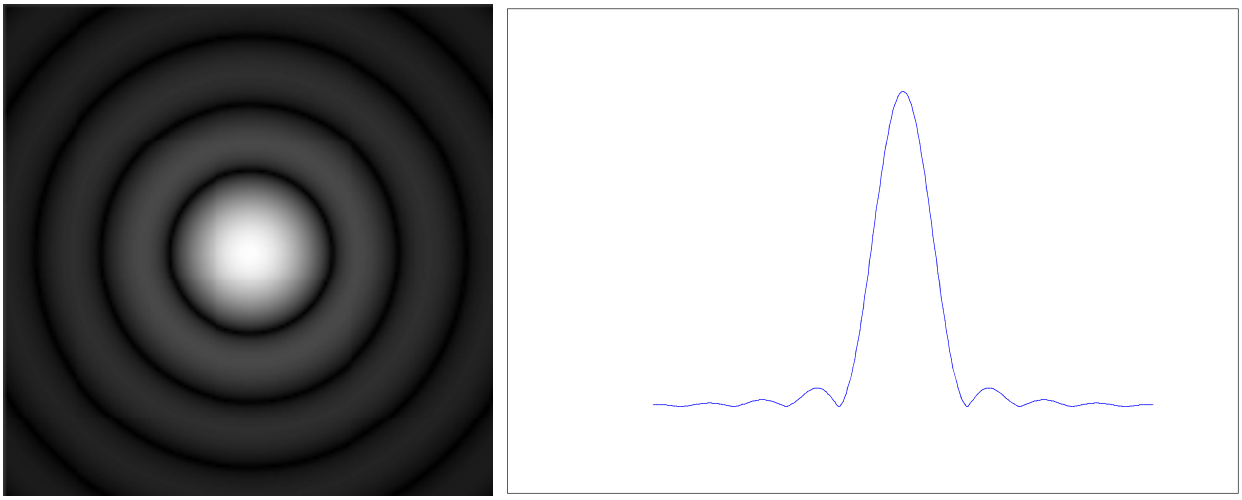

Fig. 2 Airy focus in telescopes or microscopes. This is the intensity distribution in the focal plane, if a point-shaped object is imaged. The inner disclet is separated from the outer intensity distribution by a zero. On the right side, a profile section through the middle of the pattern is shown. In both presentations, the intensity is shown in a log-scale, otherwise the structure would be difficult to recognize.

In a gedankenexperiment, we can now assume a layer of fluorochromes that have an infinite lifetime. Such fluorochromes would be excitable, but not emit fluorescence without reason. If we then illuminate that layer by means of microscope optics, the result would be a pattern of excited molecules. The pattern would represent the Airy pattern. An image very much like an Airy pattern is generated by focussing the light of a star onto a photographic film by means of a telescope. The intensity of the illumination would be translated in the ratio of excited to non-excited molecules (saturation neglected).

If the molecules would be enabeled to emit their energy in a fluorescence process, we would exactly see the Airy pattern in our fluorescence detector. If we illuminated the layer before fluorescence emission homogeneously by a spatial homogeneous flash of light with a wavelength that can initiate stimulated emission, no signal would be detectable by the fluorescence detector, Obviously, this is a nice experiment in theory, but does not improve resolution whatsoever. For that, we need to apply an optical trick (still diffraction-limited).

2.3 Badly focussed light

The focus pattern can be modified by introducing an appropriate phase plate. There are many ways to alter the shape of the focus, and many shapes can be created. For our purpose, a helical phase plate is best suited. Such plates are readily available, and not complicated to implement: they are simply put in the beam before the focussing lens. The result is a pattern in the focus plane which is not brightest in the middle, but has a zero in the centre. The brightest intensity is a ring around that central zero. For ordinary microscopy this would be a disaster, as the focus is too bad to give reasonable resolution.

If we focus light that can initiate stimulated emission onto our pattern of excited molecules, applying the helical phase plate, we would consequently not stimulate emission in the centre of the pattern. The bright ring of emission-stimulating light would return the molecules located in that ring-shaped area to the ground state. After that illumination, the initial pattern would have changed: Although the centre would still be excited, molecules around the center would have been driven into the ground state. It is obvious that the residual circle of still excited molecules would shrink by widening the ring of emission stimulating light. If done correctly, at infinitely high intensities of the emission stimulating light, the de-excitation would cover the whole pattern except for a point-like residual which corresponds with the zero of the "bad" focus.

If we then could make the residual molecules release their energy by spontaneous emission (fluorescence), the emitting pattern would be of infinitely small size, corresponding to an infinitely high resolution (diffraction-unlimited).

This is the mechanism that is employed in STED microscopy: a diffraction-limited spot of exciting light is scanned over the sample (scanning is needed in order to obtain a two-dimensional image). A toroid focus coaxially illuminates

the sample with light that initiates stimulated emission. In the first systems, the molecules were excited with a pulsed laser, and the de-excitation was performed with a pulsed laser in a synchronized manner a brief interval after the excitation pulse. As fluorochromes have very short lifetimes (in the range of a few nanoseconds), the fluorescence emission can be collected between the STED pulse and the next excitation pulse.

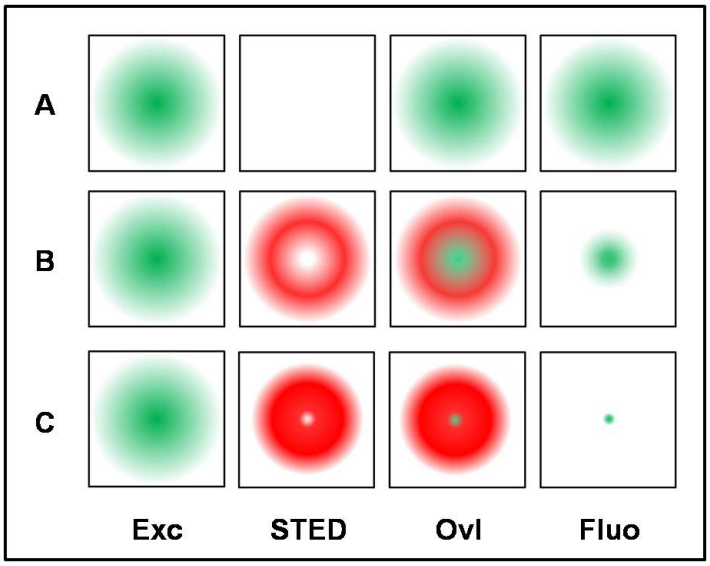

Fig. 3 Effect of various intensities of toroidally focused light that initiates stimulated emission on the residual fluorescing area. The first row shows the diffraction-limited illumination which is identical with the initial excitation pattern. The second row shows various intensities of the STED illumination with a toroid focus. A: zero intensity, B: weak and C: strong intensity. Both beams are overlaid in the third row. The residual excited areas, which are the fluorescence emitting areas, are shown in the last row.

Currently, a resolution of some 50nm is routinely achieved (which is 1/4 of the diffraction limit). Resolutions with this method down to merely 3nm, (1/70 of diffraction) have been reported, but not in typical sample situations in biomedical applications.

2.4 Further improvements

A constraint in the beginning of STED microscopy was the limitation to only a few specific fluorochromes that could fit the instrumental parameters, especially with respect to excitation and emission spectra. Meanwhile, a large range of dyes have been found or designed, and it is possible to tag biological structures by a number of fluorochromes. Also, multicolour STED and STED with fluorescent proteins is possible. This is especially advantageous, as STED is the only superresolution method that is fast enough to create meaningful time lapse experiments with living material.

An instrumental simplification was possible by using continuous wave illumination instead of pulsed lasers. CW lasers are simpler and available with many colours, which further increases the range of fluorochromes that can be used for STED. In CW STED, the balance of excitation and stimulated emission allows fluorescence emission to be collected from a region where the equilibrium favours excited states.

A combination of two-photon excitation and STED is a promising tool to increase the z-sectioning performance for STED imaging, and opens STED for thicker samples due to lower scattering(8)

The latest improvement is gated STED. This method uses detectors that can be controlled to collect only a predefined time interval between two de-excitation pulses. As the fluorescence lifetime depends on both the quantum properties of the fluorochrome and the various ways of returning from the excited state (of which stimulated emission is one), the lifetime is longest in the centre of the overlaid diffraction pattern. The fluorescence lifetime decreases radially with the intensity of the depletion laser as a consequence of additional paths for leaving the excited state. Modern instrumentation employing hybrid detectors that can be modified to collect signals only in a predefined time interval (gated mode) allows the experimenter to decide which fluorescence photon will be collected. A collection gate that covers the full interval between the pulses will collect all photons that are in the residual area. If the gate is restricted to the later part of the interval (close to the next pulse), only long-lived fluorescence states are recorded. As these occur preferentially in the centre of the pattern, the area from which emission is collected is further narrowed. This is in consequence an additional increase in resolution.

3. Ground State Depletion Microscopy

An entirely different approach for seeing structural details far below the diffraction limit was described, for example, by localizing spectrally different dyes(9) Although such localizations as single measurements do not show any resolved image, it is possible to reconstruct the structural patterns by localizing many single emitters. The resolution of such reconstructions is then defined by the precision of the localization.

3.1 Localization

As pointed out in the section above, a single spot-shaped emitter will cause a typical image: the diffraction pattern. Although it is not possible to tell the actual shape and size of the imaged object (if small enough), one can tell quite exactly the position of the emitter, which is the center of mass of the intensity in the diffraction pattern. The usual approach for finding the position of an emitter from its intensity distribution is to fit a two-dimensional Gaussian with the recorded diffraction pattern (more exactly, the fit should be done with an Airyan). The fit will yield an xy-coordinate and a variance, the latter is a measure for the precision of the localization(10)

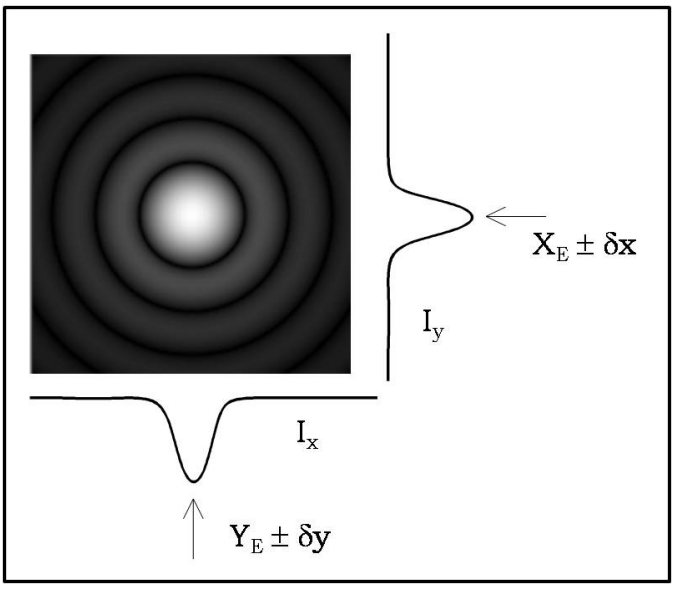

Fig. 4 Localization of a sub-diffraction limit sized emitter. Fitting a two dimensional Gaussian to the intensity distribution yields x- and y-coordinates and variances in x and y.

3.2 Separation

It is obvious that localization of an emitter is easy if the emitters have a distance of several times the diameter of the center disc of the diffraction pattern. If the emitters are very close, the patterns are overlaid when recorded simultaneously. This is what limits resolution under standard imaging conditions, no matter whether the image is taken in parallel (widefield) or sequential (scanning) mode.

The challenge is therefore to look at the emitters separately. Of course, the darkfield images of colloidal solutions, as described by Zsygmondi, feature single emitters. But this is because they are very sparsely distributed in the solution. As a consequence, they do not have or form a structure.

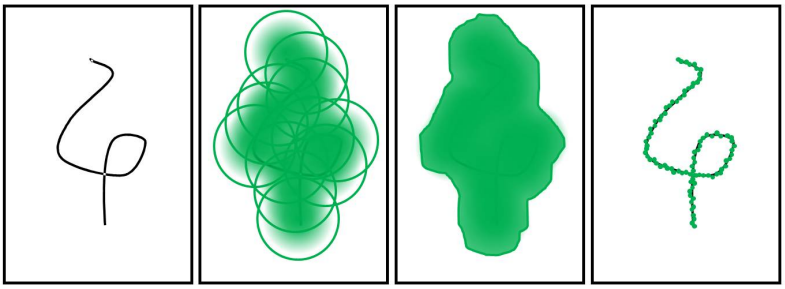

Fig. 5 From left to right: 1. Original structure, decorated with emitters. 2. Each emitter generates a diffraction pattern. 3. All emitter patterns melt and obscure the original structure. 4. Good representations by localized single emitter coordinates with small variances (shown as circles).

As mentioned above, one way of separating the emitters is to paint them with different colours. If a green and a red emitter are very close (much closer than the size of the diffraction pattern), they can be separated into different channels and the centres of mass (coordinates) can be analyzed. Separation is possible down to distances of a few nanometres. The result reveals both the distance and relative location of the emitters.

Even a small field of view contains thousands to millions of emitters. Therefore, spectral separation is very much limited to a few emitters. A better approach than spectral separation is temporal separation. This requires the emitters to switch between on and off states. The switch may be induced, which is the case for some fluorescent proteins (e.g. DRONPA)(11) or chemical dye associations (STORM)(12) Here, the sample is illuminated by a switch wavelength, with the consequence that a few emitters in the field are switched to the on-state. The art is to switch few enough, in order to ensure that each pattern is truly originating only from a single emitter. Then an image is taken. The image is analyzed for centre of intensity coordinate of each pattern. These coordinates are stored (e.g. in a new target image). Then, a second switch-illumination procedure will light up a set of different emitters, which are recorded and analyzed in the next image. If this sequence is continued for a sufficient number of times, one can approximate the decoration of the original structure with high precision. The precision depends on the brightness and stability of the emitters. It is obvious that a sufficient number of emitters must be recorded. Consequently, the process of image acquisition, analysis and data storage has to be repeated many times – in practice several thousands of times. Therefore, the acquisition of a decent superresolution image by localization will take a couple of minutes at least.

3.3 The triplet approach

A different method to obtain separated emitters in time is the usage of intrinsically switching probes. Many fluorescent proteins show stochastic switching between dark and fluorescent states, called blinking. Also, fluorescent quantum dots show this kind of behavior, which was employed for a superresolution approach by R. Heinzmann(13) The most versatile method, though, uses dark state transitions that occur in more or less any fluorescent dye typically associated with triplet states.

When an ensemble of emitters is illuminated, transitions from the ground state to the excited state and spontaneous return to the ground state cause the emission of fluorescent photons. To some extent (ruled by quantum mechanical properties of the probe), instead of emitting a fluorescent photon, the emitter can also undergo a different transition into a long-lived state, usually a triplet state. The lifetime of triplet states is in the range of microseconds to milliseconds (or even longer). Then, a spontaneous return to the ground state – usually without emitting a photon – converts the emitter to its normal fluorescent state. If the illumination is bright enough, one can transfer all emitters into the triplet state. Only occasionally, a molecule will return stochastically from the triplet state and then provide fluorescence photons for a short time before switching back to the dark triplet state. The approach is referred to as ground state depletion(14) (although more logically it is a dark-state colonization).

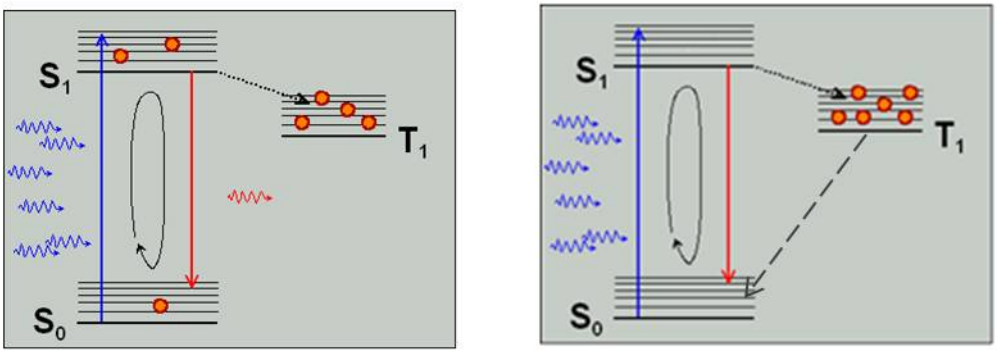

Fig. 6 Left: At high illumination intensities, many elements of an ensemble of fluorescent molecules will assume a long-lived triplet state. Right: At very high illumination intensities, most of the molecules will be transferred into the triplet state. Only occasionally a molecule will return to the ground state (dashed line) and can then for a short time cycle between ground and excited state to provide photons before switching back to the triplet state.

The short time when the emitter is transiently in the non-dark state will create an isolated diffraction pattern if the boundary conditions are set properly. The beauties of this approach are firstly no need for additional illumination schemes, just an intense enough excitation illumination. Secondly, more or less all fluorescent probes show a behavior that can be utilized in the way described above.

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