5 STED microscopy: exploring fluorescence lifetime gradients for super-resolution at reduced illumination intensities

Abstract: The 21st century has opened with the development of several strategies to push the spatial resolution of far-field fluorescence microscopy beyond the diffraction limit. Among all these super-resolved techniques, stimulated emission depletion (STED) microscopy stands out for its general principles and its fast acquisition capability. However, the application of STED microscopy to live-cell imaging has been limited by the typically high illumination powers required by early implementations of the technique. One way to preserve the effective resolution of a STED microscope, but with a significant reduction of the illumination intensity, is by using the nanosecond fluorescence dynamics information contained in a time-resolved STED experiment. Two different implementations exploring this idea have been demonstrated so far, respectively the so-called gated-STED (g-STED) microscopy and Separation of Photons by Lifetime Tuning (SPLIT)-STED microscopy. Here we discuss the common principle behind the two methods and the benefits and major differences between the two implementations.

5.1 Introduction

Since its invention in 1908, far-field fluorescence microscopy has been an essential source of major discoveries in life sciences [1, 2]. Throughout the 20th century far-field microscopy has grown enormously and all its distinctive features, such as high sensitivity and specificity through fluorescent tagging, the possibility to investigate the interior of living organisms and the ability to extract quantitative data about molecular dynamics and organizations, were continuously refined. However, the fundamental barrier limiting the spatial resolution of early far-field fluorescent microscopy, was still intact at the end of the 20th century. Namely, the diffraction barrier precluded far-field microscopy access to the entire sub-cellular components whose structural complexities were below around half the wavelength of the light used for the investigation (i.e., ≈ 200 nm).

The 21st century opened with a fundamental microscopy revolution, which saw the introduction of new viable concepts able to effectively overcome the diffraction barrier and push the spatial resolution of a far-field fluorescence microscope to the double-digit nanometer scale [3–7]. These techniques are usually referred to as super-
resolved microscopies and their importance has been recently recognized by the award of the 2014 Nobel Prize in Chemistry to their inventors.

Even though super-resolved fluorescence microscopy currently includes many different techniques [8–14], with fundamental differences in implementation and the photophysical mechanism used to circumvent the diffraction barrier, all of them rely on the same basic principle [15]. All super-resolved techniques resolve features smaller than the diffraction limit by getting the fluorophores (defining the structures) transiently into two discernible (pseudo-)states, i.e., (pseudo-)states with different spectral or temporal or any other detectable responses to the illumination. The transition between distinguishable states ensures that the signal recorded stems from fluorophores within a region of the sample whose size is much smaller than the diffraction limit. Scanning this region across the sample leads to images with subdiffraction resolution.

Among all these super-resolved techniques, stimulated emission depletion (STED) microscopy [8] stands out for its generality and its fast recordings. Indeed, STED microscopy uses two fundamental processes to transiently transfer a fluorophore from a dark state to a bright state (the fluorophore emits a detectable signal): the excitation process transfers the fluorophore from the singlet ground state (dark) to the singlet excited state (bright) and the stimulated emission (SE) process does the opposite. The use of these two fundamental processes makes, at least in theory, STED microscopy compatible with all fluorescent probes. However, at the same time the use of these transitions imposes the use of high dose of illumination intensity to effectively drive the fluorophore into the dark state. In particular, in a typical STED microscope, a focused Gaussian beam excites all the fluorophores within a diffraction-limited spot. Successively, a focused doughnut-shaped beam, the STED beam, featuring a zero-intensity point coaligned with the peak of the Gaussian excitation spot and a wavelength able to induce stimulated emission, quenches all the excited fluorophores except those located in a tiny subdiffraction sized region around the zero-intensity point. In order to efficiently quench fluorescence, the stimulated emission process has to compete with spontaneous de-excitation, which normally occurs after few nanoseconds ($\tau_0 \approx 1–10$ ns) from the excitation event. This short time window in which stimulating photons have to act, together with the low cross section of the stimulated emission process ($\sigma_{\text{STED}} \approx 10^{-17}–10^{-16}$ cm$^2$) [16], demand a high dose of illumination intensity from the STED beam, i.e., a STED microscope normally operates with intensities of the order of 0.1–1 GW/cm$^2$. Such a range of intensities might introduce side effects such as photobleaching of the fluorophores and phototoxicity for the sample. As a consequence, STED microscopy was long thought to be incompatible with live-cell recording and long-term imaging and a prominent part of the developments in the field of STED microscopy has been focused on the mitigation of this drawback.

Two synergetic strategies have been followed. The first strategy aims at the development of new fluorescent probes with high photostability and/or with spectral
fingerprint in the near-infrared region, where phototoxicity reduces [17, 18]. The second strategy aims at the reduction of illumination intensity without sacrificing the spatial resolution and without changing the basic mechanisms of STED microscopy [19, 20].

Within this latter strategy, it has been demonstrated that using the nanosecond fluorescence dynamics information of a time-resolved STED microscopy experiment it is possible to improve the effective resolution of STED microscopy without increasing the (peak) intensity of the STED beam or, equivalently, to reach a certain subdiffraction resolution with reduced (peak) intensity [19, 21]. Two different implementations, but exploring the same idea and working on similar data sets, have been demonstrated so far, respectively the so-called gated-STED (g-STED) microscopy [19, 22–24] and separation of photons by lifetime tuning STED (SPLIT-STED) microscopy [20].

Gated-STED and SPLIT-STED microscopy are based on the observation that since the stimulated emission process opens a new de-excitation pathway for the excited fluorophore, it also shortens the excited-state lifetime of the fluorophore, i.e., the time that the fluorophore spends on average in the excited state after the excitation event. In particular, if the STED beam is running in continuous wave (CW) the lifetime of the fluorophore decreases as

\[
\tau_{SI} = \frac{1}{k_0 + k_{STED}}
\]

with

(i) \( k_0 = 1/\tau_0 \) the rate of spontaneous de-excitation, which is the inverse of the excited-state lifetime \( \tau_0 \) in the absence of the STED beam;

(ii) \( k_{STED} \propto I_{STED}^* \) the rate of stimulated emission, which is directly proportional to the instantaneous intensity \( I_{STED}^* \) of the STED beam [25].

Consequently, the doughnut-shape of the STED beam imposes a spatial signature for the excited-state lifetime of the fluorophores within the excitation spot, i.e., the excited-state lifetime of a fluorophore changes according to its position. The effective excited-state lifetime decreases away from the zero-intensity point, reaching a minimum in the proximity of the doughnut crest where there is maximum STED beam intensity.

As a matter of fact, by selecting only those photons that stem from the long-lived fluorophores it is possible to further reduce the volume from which the registered signal is generated, thus further increasing the resolution of the STED system, without increasing the intensity of the STED beam. It is important to highlight that, since the intensity of the beam does not increase, fluorescence quenching does not improve and the effective fluorescent volume does not change: it is only the region from which the fluorescence signal is recorded that decreases.

The gated-STED and the SPLIT-STED microscopes vary according to the method they use to select the photons from the long-lived fluorophores, which reside in the central part of the excitation volume. However, the two implementations share similar STED architecture. In particular, both need access to the so-called photon arrival time information, namely, for each fluorescent photon it is necessary to know the time passed from the excitation event that generated the photon. This is normally obtained
using a pulsed laser which synchronizes the excitation events and a dedicated electronic that measures (with a precision of few tens of picoseconds) the arrival time of the photons.

Given the photon arrival times for each pixel the gated-STED microscope simply selects the photons within a certain time window (time-gated detection) [19], whilst the SPLIT-STED microscope performs a phasor-based analysis on the full photon arrival time histogram [20].

In this chapter we discuss the benefits and the major differences of the two implementations.

5.2 Gated- and SPLIT-STED theory

5.2.1 Temporal point spread function

Both gated-STED and SPLIT-STED microscopy decode extra spatial information from the nanosecond temporal dynamics of the fluorescence signal. In particular, both methods take advantage of the spatial signature of the excited-state lifetime induced by the SE process. Indeed, fluorescence photons are emitted with different temporal dynamics according to the position of the fluorophore inside the STED detection volume. Roughly speaking, photons emitted from the center of the detection volume are characterized by a slow dynamics, whilst photons emitted from the periphery follow fast dynamics. It is clear that both methods improve spatial resolution by highlighting photons that originate from the center and discarding photons that originate from the periphery.

The common starting point for a theoretical description of both approaches is the characterization of the temporal variation of the fluorescent detection volume of the STED system. Since we are mainly describing the detection volume in an imaging system, we can also refer to it with the term point spread function (PSF). For the sake of clarity we will limit the analysis to the lateral dimensions (bi-dimensional model). To obtain an analytical description of the temporal PSF we make the following assumptions:

- a parabolic approximation for the doughnut-shaped STED beam

\[ I_{\text{STED}}(r) = I_{\text{STED}}(w) r^2 / w^2, \]

with \( I_{\text{STED}}(w) \) the STED beam intensity at position \( r = w \);

- a Gaussian profile of the confocal PSF \( h(r) = \exp\left(-2r^2/w^2\right) \), with \( w \) being the beam waist along the radial direction and \( r^2 = x^2 + y^2 \) the radial distance from the focal point (\( x = 0, y = 0 \));

- a single exponential decay rate for the unperturbed fluorophores \( y_0 = 1/\tau_0 \), where \( \tau_0 \) is the unperturbed excited-state lifetime;
the fluorophores are excited at time zero \((t = 0)\) and the STED beam is active during the entire time course of the experiments (i.e., the STED beam is running in CW).

The instantaneous probability of stimulated emission depends linearly on the STED beam intensity:

\[
\gamma_{\text{STED}}(r^2) = \gamma_0 \left[ \frac{I_{\text{STED}}(r)}{I_{\text{SAT}}} \right], \tag{5.1}
\]

where the constant \(I_{\text{SAT}}\), usually called saturation intensity, represents the value of intensity for which \(\gamma_{\text{STED}} = \gamma_0\). The resulting radial distribution of the decay rates can be approximated by a parabolic function:

\[
\gamma(r^2) = \gamma_0 + \gamma_{\text{STED}}(r^2) = \gamma_0 + \gamma_0 \left[ \frac{I_{\text{STED}}(w)^2}{I_{\text{SAT}}} \right] \frac{r^2}{w^2} = \gamma_0 + \gamma_0 k_S r^2 / w^2, \tag{5.2}
\]

where we have defined:

\[
k_S = \frac{I_{\text{STED}}(w)}{I_{\text{SAT}}}. \tag{5.3}
\]

According to this definition, \(k_S\) is the ratio between \(I_{\text{STED}}(w)\) and the saturation value \(I_{\text{SAT}}\) for which the probability of decay due to SE and spontaneous emission are equal.

**Fig. 5.1:** (a) A doughnut-shaped STED beam coaligned with a confocal spot generates a continuous distribution of dynamics within the PSF. (b) The STED beam intensity determines the relative variation of decay rate \(\gamma / \gamma_0\) (solid line) across the PSF (dashed line). (c) Simulated time-resolved STED image of filamentous structures resembling cytoskeletal networks. The input parameters were: \(\tau_0 = 3.1\) ns, \(k_S = 12.7, S = 120, B = 0.5\). (d) The average STED decay is fitted to equation (5.11) to obtain the parameter \(k_S\) (\(k_S = 12.5\) from the fit in keeping with the input).
Importantly, the value of $k_S$ quantifies the relative variation of decay rate values within the PSF of the CW-STED microscope [20] (Fig. 5.1 (b)).

The time-dependent fluorescence intensity $F(x, y, t)$ at each pixel can be expressed as:

$$F(x, y, t) = K \int e^{-\gamma(r'^2)t} \rho(x', y') e^{-2r'^2/w^2} \, dx' \, dy',$$  

where $K$ is a constant that depends on the quantum yield of the fluorophore, the maximum of the excitation intensity and the detection efficiency, $r^2 = (x' - x)^2 + (y' - y)^2$ and $\rho(x, y)$ is the density of fluorophores.

Since the point spread function of an optical system is defined as the image of an ideal point source, we can obtain the equation of the temporal PSF by substituting the density of fluorophores $\rho(x', y')$ with Dirac’s $\delta$ function in equation (5.4). The temporal PSF reads:

$$h_{\text{STED}}(r, t) = K e^{-\gamma(r^2)t} e^{-2r^2/w^2}.$$  

### 5.2.2 Gated-STED microscopy

The gated-STED microscope increases the spatial resolution of a CW-STED implementation by discarding early photons, most likely generated from short-lived fluorophores located in the periphery of the detection volume, and highlighting late photons most likely generated from long-lived fluorophores located in the center of the detection volume. In a nutshell, the temporal filter allows to further reduce the effective detection volume. For this purpose, fluorescent photons collected within a short time interval from the excitation events $[0, T_g]$ are discarded whilst fluorescent photons in the remaining part of the excitation pulse period $[T_g, T]$ generate the final image. The point spread function of this imaging modality can be derived from the temporal integration of equation (5.5) in the interval of collection $[T_g, T]$. If we assume that the pulse interval is much longer than the excited-state lifetime of the fluorophores ($T \gg \tau_0$), the PSF of a gated CW-STED microscope reads:

$$h_{\text{g,CW-STED}}(r) = \int_{T_g}^{T} h_{\text{STED}}(r, t) \, dt$$

$$= K e^{-2r^2/w^2} \frac{1}{\gamma_0 + y_0 k_S r^2/w^2} e^{-\gamma_0 + y_0 k_S r^2/w^2} T_g$$

$$= K e^{-\gamma_0 T_g} e^{-2r^2/w^2} \frac{1}{\gamma_0 + y_0 k_S r^2/w^2} e^{-T_g \gamma_0 k_S r^2/w^2}.$$  

Notably, the effective PSF can be divided into four terms:

(i) a constant term that denotes the amplitude of the PSF, clearly indicating that the signal decreases exponentially as the time-gating value $T_g$ increases;

(ii) a Gaussian function that denotes the confocal PSF;
(iii) a Lorentzian function that reflects the PSF of a non-gated \((T_g = 0)\) CW-STED microscope;
(iv) a Gaussian term that reflects the reduction of the effective PSF for increasing \(T_g\).

The PSF (equation (5.6)) immediately highlights the positive and negative aspects of the gCW-STED implementation. For increasing \(T_g\) the effective PSF shrinks in size, furthermore the Gaussian term dominates over the Lorentzian term removing the PSF tails which drastically decrease the contrast, thus the effective resolution in the conventional CW-STED microscope. The resolution of a gated CW-STED microscope theoretically increases to infinity for increasing \(T_g\), but the reduction of the PSF’s amplitude poses an effective limit. Indeed, the reduction of the PSF’s amplitude translates into a reduction of the signal-to-noise ratio (SNR) of the gated images. It is clear that a strong reduction of the SNR can cancel out the benefits of the time-gating. In real life, the value \(T_g\) is settled to half of the excited-state lifetime \(\tau_0\), in this case the image contrast effectively improves, but the PSF’s amplitude is reduced only by a factor of 0.4. However, recently it has been shown that the SNR ratio in gated-STED implementation can be recovered by combining the usually discarded early photons with the conventional gated-STED image (late photons) through a multi-image deconvolution algorithm [26, 27].

### 5.2.3 SPLIT-STED microscopy

The goal of SPLIT is to achieve super-resolution by an explicit separation of the different components of the fluorescent signals according to their “temporal decays”. In particular, SPLIT microscopy isolates the slow component that is most likely induced by fluorophores in the center of the effective detection volume of a CW-STED system. Thus, the maximum achievable spatial resolution will be ultimately determined by the ability to distinguish between different temporal dynamics. In this framework the problem of resolving spatial features is translated into the spectroscopy problem of resolving the stimulated emission-induced variations of the fluorescence lifetime. First, we will find an explicit expression for the temporal decay of fluorescence emitted from the center of the detection volume in a CW-STED microscope. Second, we will describe a fit-free analysis of the fluorescence decays aimed at quantifying the relative contribution of this component at each pixel of the image.

#### 5.2.3.1 Explicit formulas for the fluorescence decays in CW-STED microscopy

Starting from equation (5.4), we conveniently switch to a system of polar coordinates centered on the pixel \((x, y)\) and integrate along \(\phi'\):

\[
F(x, y, t) = K \int_{0}^{\infty} C(r^2) \, dr^2 \, e^{-y(r^2)t} \, e^{-2r^2/w^2},
\]

(5.7)
With this definition, \( C(r) \) describes the effective concentration of fluorophores in a concentric ring of radius \( r \) around the pixel position. The formula shows that the temporal dynamics of \( F(x, y, t) \) encodes nanoscale spatial information in the distribution of exponential decay components. This distribution is fully described in this model by the parameters \( y_0 \) and \( k_S \) in equation (5.2).

The parameter \( \tau_0 = 1/y_0 \) depends on the specific fluorophore and can be measured from the sample with the same instrumentation by setting the STED beam power to zero. The parameter \( k_S = I_{\text{STED}}(w)/I_{\text{SAT}} \) is proportional to the STED beam power and its precise value depends on the optical configuration (in particular the shape of the excitation and the STED beams) and on the properties of the sample. We can show that, using our analytical model of SE-induced lifetime variations, we can estimate the value of \( k_S \) from the very same image \( F(x, y, t) \) by considering the average time-resolved decay \( I(t) \) of all the pixels of an image:

\[
I(t) = \iint_{x,y} F(x, y, t) \, dx \, dy.
\] (5.9)

For simplicity, consider the case of an image obtained by scanning around a point-like object, for which \( C(r^2) \approx \delta(r^2) \):

\[
\iint_{x,y} F(x, y, t) \, dx \, dy = K \iint_{x,y} \iint_0^\infty C(r^2) \, dr^2 e^{-y(r^2)t} e^{-2r^2/w^2} \, dx \, dy \\
\approx K \int_0^\infty dr^2 e^{-y(r^2)t} e^{-2r^2/w^2} \\
\propto e^{-\gamma_0 t} \frac{1}{1 + k_S y_0 t/2}.
\] (5.10)

If we take into account the presence of uncorrelated background we obtain the following functional form for the average fluorescence decay under CW-stimulated emission [20]:

\[
I(t) = A e^{-\gamma_0 t} \frac{1}{1 + k_S y_0 t/2} + B.
\] (5.11)

In order to assess the generality of this formula for the imaging of non-point-like structures, we simulated time-resolved STED images of filamentous structures similar to those found in cytoskeletal networks (Fig. 5.1(c)). The images were obtained by assuming an unperturbed fluorescence lifetime value \( \tau_0 = 3.1 \) ns whereas the STED intensity level was such that the variations across the PSF could be modeled by a value \( k_S = 12.7 \) according to equation (5.2). The resulting time-resolved STED image can be
seen as the convolution of the object with a spatio-temporal PSF (equation (5.4)). As expected, when we look at the average temporal decay of all the pixels of the image (Fig. 5.1(d)), this is not a single exponential. Here, the nonexponential behavior is not due to the presence of several fluorescent species (we are assuming a single fluorescent species with a specific \( \tau_0 \)) but rather to the continuous distribution of decay rates induced by the STED beam. The nonexponential temporal trend derived in our model and described by equation (15.1) can be a good approximation of the average STED decay (Fig. 5.1(d)). If the average STED decay is fitted to equation (5.11) we obtain the parameters \( \tau_0 = 3.1 \text{ ns} \) and \( k_S = 12.5 \) in keeping with the input. It is worth mentioning that with this analysis we are able to estimate experimentally the parameters \( \tau_0 \) and \( k_S \) of the spatio-temporal PSF without needing to know the exact value of the waist \( w \). This is because in our model the parameter \( k_S \) has been defined as an indicator of the relative variation of decay rate across the confocal PSF.

Now that we have a quantitative model for describing the SE-induced lifetime variations under the CW-STED microscopy architecture, we need a method to isolate the longer lifetime components of the distribution (Fig. 5.1(a)) in order to produce a higher resolution image. It is convenient to approximate the continuous distribution of decays to a discrete number \( n \) of decay components. Consider for instance the simplest case \( n = 2 \) (Fig. 5.2(a)). In order to find an explicit expression for the associated temporal decays (Fig. 5.2(b)), we split the integral in equation (5.10) into \( n = 2 \) parts:

\[
F(x, y, t) = K \int_0^{r_2} C(r^2) \, dr^2 \, e^{-\gamma(r^2)t} \, e^{-2r^2/w^2} + K \int_{r_1}^{\infty} C(r^2) \, dr^2 \, e^{-\gamma(r^2)t} \, e^{-2r^2/w^2}. \tag{5.12}
\]

Within this approximation, we need to define two components which do not depend on the function \( C(r^2) \). By approximating, \( C(r^2) \approx C'_0 \), we can write, for \( i = 1, 2 \):

\[
\int_{r_{i-1}}^{r_i} C(r^2) \, dr^2 \, e^{-\gamma(r^2)t} \, e^{-2r^2/w^2} \approx C'_0 \int_{r_{i-1}}^{r_i} dr^2 \, e^{-\gamma(r^2)t} \, e^{-2r^2/w^2}, \tag{5.13}
\]

where \( r_j = 0, r_1, \infty \), for \( j = 0, 1, 2 \), respectively. We are approximating \( C(r^2) \) inside \((r_{i-1}^2, r_i^2)\) with its average value within this interval and ignoring its variations inside \((r_{i-1}^2, r_i^2)\). The boundary \( r_1 \) of the two subdiffraction volumes can be arbitrarily chosen in such a way that:

\[
\int_0^T I_1(t) \, dt = \int_0^T I_2(t) \, dt. \tag{5.14}
\]

Then we define the time-dependent decay of the \( i \)-th component as:

\[
I_i(t) = \int_{r_{i-1}}^{r_i} dr^2 \, e^{-\gamma(r^2)t} \, e^{-2r^2/w^2}
\]

\[
\propto e^{-\gamma_0 t} \frac{1}{1 + k_S \gamma_0 t/2} \left( e^{-(1+k_S\gamma_0 t/2)2r_{i-1}^2/w^2} - e^{-(1+k_S\gamma_0 t/2)2r_i^2/w^2} \right). \tag{5.15}
\]
Fig. 5.2: (a) The STED-induced continuous distribution of decay rates across the PSF is approximated by only 2 spatial components. (b) The measured fluorescence decay (dashed line) is expressed as the linear combination of the slower decay component associated to the inner part of the PSF (1) and the faster decay component associated to the outer part of the PSF (2). (c) The decays are represented as vectors in the phasor plot: the experimental phasor $P(x, y)$ is assumed to be a linear combination of the phasors $P_1$ and $P_2$ plus the phasor of the uncorrelated background ($P_{BKGD}$). (d) The fractional contribution of each component ($P_1$, $P_2$, $P_{BKGD}$) is calculated and the photons are sorted into three separate images. The SPLIT image associated to the slower component has higher spatial resolution than the STED image and is separated from the background.

Then we can write the intensity as a linear combination of components:

$$F(x, y, t) \approx K \left[ C_0^1(x, y)I_1(t) + C_0^2(x, y)I_2(t) \right]$$  \hspace{1cm} (5.16)

The intensity decay at each pixel (Fig. 5.2(b), dashed line) can be expressed as the sum of a slow (Fig. 5.2(b), green line) and a fast (Fig. 5.2(b), red line) decay component, associated respectively to the fluorophores located on the inner and outer part of the PSF (Fig. 5.2(a)).

### 5.2.3.2 Phasor analysis in SPLIT-STED

Now, we will describe a fit-free analysis of the fluorescence decays aimed at quantifying the relative contribution of a specific component at each pixel of an image. This analysis is based on the phasor approach to FLIM [28–30], a methodology that has found application in FRET [31–35], spectral fingerprinting [36, 37], correlation spectroscopy [38, 39] and super-resolution [20]. An important outcome of the whole SPLIT-STED approach is that any contribution to the fluorescence signal that is uncorrelated on the nanosecond timescale is separated from the final super-resolved image.
For example, the anti-Stokes fluorescence emission induced by the STED beam. Notably, this source of background will be uncorrelated since the STED beam is running in CW. Anti-Stokes fluorescence emission is a common source of background in STED microscopy, especially when the STED beam’s wavelength is closer to the emission maximum of the fluorophore [40, 41].

In the phasor analysis any decay \( J(t) \) is converted via a Fast Fourier Transform (FFT) into a pair of phasor coordinates \( (g, s) \) defined as:

\[
\begin{align*}
g &= \frac{\int_0^T J(t) \cos(2\pi t/T) \, dt}{\int_0^T J(t) \, dt}, \\
s &= \frac{\int_0^T J(t) \sin(2\pi t/T) \, dt}{\int_0^T J(t) \, dt},
\end{align*}
\]

(5.17)

where \( T \) is the pulse interval. Here we assume that \( T \gg \tau_0 \), thus the function \( J(t) \) has already decayed to background [30]. The slow and fast decays (Fig. 5.2(b)) can be now represented as two vectors \( \mathbf{P}_1 = (g_1, s_1) \) and \( \mathbf{P}_2 = (g_2, s_2) \) in the phasor plot (Fig. 5.2(c)).

We can write the intensity at one pixel as the sum of two components plus a term representing any uncorrelated background \( F(x, y, t) = A_1(x, y)I_1(t) + A_2(x, y)I_2(t) + I_{BKGD}(x, y) \). Thus, the phasor of each pixel \( \mathbf{P}(x, y) = (g_1(x, y), s_1(x, y)) \) can be expressed as a linear combination of the phasors of the two components and the phasor of the background

\[
\begin{align*}
g(x, y) &= \frac{\int_0^T [A_1(x, y)I_1(t) + A_2(x, y)I_2(t) + I_{BKGD}(x, y)] \cos(2\pi t/T) \, dt}{\int_0^T [A_1(x, y)I_1(t) + A_2(x, y)I_2(t) + I_{BKGD}(x, y)] \, dt} \\
&= \frac{N_1(x, y)}{N(x, y)} g_1 + \frac{N_2(x, y)}{N(x, y)} g_2, \\
s(x, y) &= \frac{\int_0^T [A_1(x, y)I_1(t) + A_2(x, y)I_2(t) + I_{BKGD}(x, y)] \sin(2\pi t/T) \, dt}{\int_0^T [A_1(x, y)I_1(t) + A_2(x, y)I_2(t) + I_{BKGD}(x, y)] \, dt} \\
&= \frac{N_1(x, y)}{N(x, y)} s_1 + \frac{N_2(x, y)}{N(x, y)} s_2,
\end{align*}
\]

(5.18)

where the total number of photons \( N(x, y) \) detected at one pixel is the sum of the photons originating in the two spatial components plus the uncorrelated background \( N(x, y) = N_1(x, y) + N_2(x, y) + N_{BKGD}(x, y) \). Since the uncorrelated background is independent of \( t \), its phasor coordinates are \( \mathbf{P}_{BKGD} = (0, 0) \) (Fig. 5.2(c)). Using vector notation we can rewrite:

\[
\mathbf{P}(x, y) = \frac{N_1(x, y)\mathbf{P}_1 + N_2(x, y)\mathbf{P}_2}{N(x, y)} = f_1(x, y)\mathbf{P}_1 + f_2(x, y)\mathbf{P}_2.
\]
This linear system of equations can be written in the form \( P = Mf \), where \( f = (f_1, f_2) \) is the vector of the unknown fractional components and \( M_{ij} \) is the matrix

\[
M = \begin{pmatrix} g_1 & g_2 \\ s_1 & s_2 \end{pmatrix},
\]

(5.19)

which describes the two different temporal dynamics in the phasor domain. The solution of this system is given by \( f = M^{-1}P \). The images \( N_i(x, y) (i = 1, 2) \) of the photons emitted in each of the two subdiffraction volumes and the image \( N_{\text{BKGD}}(x, y) \) of the background can be calculated as \( N_i(x, y) = f_i(x, y)N(x, y) \) and

\[
N_{\text{BKGD}}(x, y) = \left[ 1 - \sum_{i=1}^{n} f_i(x, y) \right] N(x, y),
\]

(5.20)

respectively (Fig. 5.2(d)). By using a model for the STED-induced variations of fluorescence lifetime and the phasor analysis of lifetime data we have obtained a super-resolved image \( N_1(x, y) \) characterized by a higher effective resolution than the CW-STED counterpart (Fig. 5.2(d)).

Extending this procedure to \( n \) components we can state that the original image \( N(x, y) \) can be split into \( n + 1 \) images based on the assumption that we are able to distinguish, within our observation volume, \( n \) different dynamics, associated with \( n \) linearly independent vectors in the \( n \)-dimensional phasor space. If we take noise into account, we find that the larger the number \( n \) of components, the higher will be the noise propagated to the final SPLIT images. Indeed, since the SPLIT image is calculated as: \( N_1(x, y) = f_1(x, y)N(x, y) \), it is affected by the noise in the STED image \( N(x, y) \) and, in addition, by any noise in the factor \( f_1(x, y) \). The factor \( f_1(x, y) \) is calculated at each pixel from the measurement of \( g(x, y) \) and \( s(x, y) \) which contain experimental noise. The noise in the measurement of \( g \) and \( s \) (or higher harmonics components) is propagated to the fraction \( f_1 \) through the linear system \( P = Mf \) depending on the matrix \( M \). One way to quantify the error propagation through this linear system is by considering the condition number \( k_{\text{cond}} \) defined as the product of the norms of the matrices \( M \) and its inverse \( M^{-1} \),

\[
k_{\text{cond}} = \|M\| \|M^{-1}\|.
\]

This parameter decreases with higher values of \( k_S \) and increases exponentially with the value of \( n \) [20]. Even if, theoretically, unlimited spatial resolution could be foreseen by considering higher values of \( n \), we must conclude that, for practical purposes, noise will restrict the use of the SPLIT algorithm only to low values of \( n \).

### 5.3 Gated- and SPLIT-STED comparison

The operational difference between gated-STED and SPLIT-STED is that, for each pixel, the gated-STED microscope simply selects the photons within a certain time window (time-gated detection), whereas the SPLIT-STED microscope performs a phasor-based
5.3 Gated- and SPLIT-STED comparison

This operation is aimed at isolating the slower component corresponding to the inner part of the PSF. However, there is also a strong conceptual difference between these two approaches: whilst the SPLIT-STED approach attempts to perform a true separation of the dynamics components, the gated-STED approach selects the fluorescence photons that most likely belong to the slow dynamics (the longer $T_g$ is, the higher is the probability that the registered photons belong to the slow dynamics and thus are generated from the center of the detection volume). On the other hand, time-gating is straightforward: it does not require the data analysis involved in the SPLIT framework and is simple to implement. In particular, since it does not need the full photon arrival time histogram, the expensive time-correlated single-photon counting based hardware can be substituted by cheaper time-gated electronics [19, 23, 42].

The difference between the two approaches can be more easily understood by looking at a specific experimental case. Fig. 5.3 (a) and (c) report the time-resolved confocal and STED images of an area of a fixed cell where microtubules have been immunostained with the dye Oregon Green 488. The phasor plot of the confocal image (Fig. 5.3 (b)) shows a single spot in agreement with the fact that all the pixels of the image have the same lifetime. The phasor plot of the STED image (Fig. 5.3 (d)) shows a significant spreading, indicating that the pixels of the image may have different lifetimes. Remarkably, there is a significant bending of the phasor toward the

![Figure 5.3](image)

**Fig. 5.3:** Time-gating versus SPLIT analysis in time-resolved STED. Time-resolved confocal (a) and STED (c) images of tubulin in fixed HeLa cells immunostained with Oregon Green 488 and associated phasor plots (b, d). (e) Time-gated image ($T_g = 1$ ns) and (f) sketch of the operation of time-gating in the time domain. (g) SPLIT image series showing the images associated respectively to the slow component (1), fast component (2) and the uncorrelated background (BKGD). (h) Sketch of the operation of SPLIT in the time domain. The STED beam power was set to 40 mW. Scale bar 2 µm.
origin of the plot indicating the presence of a strong uncorrelated background in most of the pixels. The presence of uncorrelated background is explained by the anti-Stokes fluorescence emission induced by the CW-STED beam at $\lambda = 560$ nm on the dye Oregon Green 488 [20, 43]. In this case, when we perform time-gating, we efficiently reject the fast temporal component but the signal is still degraded by the contribution from the background (Fig. 5.3 (f) and (g)). Since the background is not separated from the high-resolution component, the final time-gated image does not show a significant improvement in spatial resolution (Fig. 5.3 (f)). On the other hand, by performing the SPLIT analysis, we are able to generate three separate images (Fig. 5.3 (g)) corresponding to the slower temporal component (1), the faster temporal component (2) and the background (BKGD). In this case the super-resolved SPLIT image is not degraded by the presence of the strong uncorrelated background signal (Fig. 5.3 (g)). This is because the SPLIT image represents the exclusive contribution of each temporal component to the total signal in a pixel (Fig. 5.3 (h)). In other words, only with the SPLIT approach are we performing a true separation of the dynamics components at each pixel of the image. It is worth noting that different approaches exist to remove the anti-Stokes emission background from gated-STED. In TCSPC-based gated-STED implementation, the anti-Stokes emission background can be subtracted/removed by estimating it from the photon arrival times histogram [27, 43], similar to SPLIT-STED. More generally, anti-Stokes emission background can be subtracted by using lock-in (synchronous) detection approaches [40].

5.4 Discussion and conclusions

We have presented two methods, gated-STED and SPLIT-STED, that provide a significant reduction of illumination intensity, without sacrificing spatial resolution and without changing the basic mechanisms of STED microscopy. Both methods exploit the nanosecond fluorescence dynamics information of a time-resolved STED microscope to improve the effective spatial resolution without increasing the (peak) intensity of the STED beam or, equivalently, to reach a certain subdiffraction resolution with reduced (peak) intensity. The main difference between the two approaches resides on the strategy they use to select the photons from the long-lived fluorophores residing in the central part of the fluorescent volume: the gated-STED microscope simply selects the photons within a certain time window (time-gated detection), while the SPLIT-STED microscope performs an explicit analysis of the temporal dynamics of fluorescence.

It is worth noting that this strategy of improving spatial resolution by using differences in fluorescence dynamics is common to other techniques that are not based on stimulated emission. For instance DSOM [44], which exploits a singlet-triplet state transition [45], and methods based on the switching dynamics of reversibly switchable fluorophores [46], also take advantage of the additional spatial information encoded
in fluorescence dynamics. However, the timescale on which the dynamics is recorded depends on the photophysical process exploited by the technique. For instance, in the aforementioned methods it ranges from microseconds (DSOM) to milliseconds (reversible switching), which is much slower than what can be achieved in SPLIT-STED. Since in the latter case the dynamics is recorded in the nanosecond range, the maximum achievable temporal resolution of SPLIT-STED is not compromised and this approach is also compatible with those techniques, like fluorescence correlation spectroscopy (FCS) [47], that require temporal resolution down to the microsecond scale.

As a matter of fact, FCS has become one of the methods of choice to probe the motion of proteins and other molecules with minimal perturbation on specific subcellular compartments [48–50]. An efficient combination of FCS with super-resolution microscopy would represent the opportunity to probe molecular diffusion at the nanoscale inside cells. STED-FCS has been successfully applied to study the dynamics of membrane lipids at the nanoscale [51, 52]. In the context of live-cell experiments, the use of strategies aimed at reducing illumination intensity, without sacrificing spatial resolution, is of crucial importance. For this reason we expect that the principles of gated-STED and SPLIT-STED will be exploited in the near future also in this field [53].

References


