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[Microscopy beyond imaging: space-resolved photochemistry and photobiology](#)

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Editorial

[Microscopy beyond imaging: space-resolved photochemistry and photobiology](#)

F. de Schryver and S. Nonell, *Photochem. Photobiol. Sci.*, 2009, **8**, 441

Perspective

[Photosensitized production of singlet oxygen: spatially-resolved optical studies in single cells](#)

T. Breitenbach, M. K. Kuimova, P. Gbur, S. Hatz, N. B. Schack, B. W. Pedersen, J. D. C. Lambert, L. Poulsen and P. R. Ogilby, *Photochem. Photobiol. Sci.*, 2009, **8**, 442

Communication

[Towards direct monitoring of discrete events in a catalytic cycle at the single molecule level](#)

R. Ameloot, M. Roeffaers, M. Baruah, G. De Cremer, B. Sels, D. De Vos and J. Hofkens, *Photochem. Photobiol. Sci.*, 2009, **8**, 453

Papers

[Spatial and temporal dynamics of *in vitro* photodynamic cell killing: extracellular hydrogen peroxide mediates neighbouring cell death](#)

N. Rubio, S. P. Fleury and R. W. Redmond, *Photochem. Photobiol. Sci.*, 2009, **8**, 457

[Multicolor photoswitching microscopy for subdiffraction-resolution fluorescence imaging](#)

S. van de Linde, U. Endesfelder, A. Mukherjee, M. Schüttpelz, G. Wiebusch, S. Wolter, M. Heilemann and M. Sauer, *Photochem. Photobiol. Sci.*, 2009, **8**, 465

[Multiparameter fluorescence image spectroscopy to study molecular interactions](#)

S. Weidtkamp-Peters, S. Felekyan, A. Bleckmann, R. Simon, W. Becker, R. Kühnemuth and C. A. M. Seidel, *Photochem. Photobiol. Sci.*, 2009, **8**, 470

[Triplet-relaxation microscopy with bunched pulsed excitation](#)

G. Donnert, C. Eggeling and S. W. Hell, *Photochem. Photobiol. Sci.*, 2009, **8**, 481

[Single-molecule photophysics of oxazines on DNA and its application in a FRET switch](#)

J. Vogelsang, T. Cordes and P. Tinnefeld, *Photochem. Photobiol. Sci.*, 2009, **8**, 486

Triplet-relaxation microscopy with bunched pulsed excitation

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Obtaining high signal levels in fluorescence microscopy is usually spoiled by the concomitant population of the dark (triplet) state of the marker, which is often followed by photobleaching. Recently, we introduced the triplet relaxation (T-Rex) modality in fluorescence microscopy which led to a major increase in total signal and dye photostability. The idea behind T-Rex is to avoid the illumination of fluorophores in the triplet state, *e.g.* by using pulsed excitation with interpulse time distances that are long enough for the triplet state to relax between two pulses. While our previous implementation came at the expense of extended recording, here we investigate pulsed excitation patterns for T-Rex illumination implying shorter total recording times. In particular, we balance signal enhancement and imaging speed by exciting with bunches of quickly succeeding pulses that are separated by dark periods for triplet relaxation. Taking the green fluorescent protein and the organic dye Atto532 as examples, we observe the dependence of photobleaching and total fluorescence gain on the number of pulses within a bunch. Reaching almost T-Rex conditions this excitation scheme mimics fast scanning of the illumination beam and has the potential to improve a whole range of analytical tools that suffer from photobleaching and low signal levels.

Introduction

Fluorescence microscopy is firmly established in a wide range of scientific applications. Tagging the sample of interest with fluorescent markers, the fluorescence readout can expose spatial and temporal distributions of molecules with large specificity and detail.¹ Nevertheless two key properties of standard microscopy, namely the spatial resolution and the limited signal, call for major improvements.² Addressing the latter, triplet-relaxation (T-Rex) microscopy has demonstrated a substantial gain in fluorescence detection.^{3,4} The T-Rex concept is based on the fact that the fluorescence marker's long-lived dark (triplet) state⁵ restricts the fluorescence emission, both because the dye is trapped in the dark state and dislodged from excitation-emission cycling during this period, and because the triplet state is a major gateway for irreversible photobleaching reactions. For example, additional excitation from the lowest to higher electronically excited triplet states opens up very efficient reaction channels.^{6–8} Allowing for relaxation of the triplet state between subsequent excitation pulses, the T-Rex mode improved the photostability and increased the fluorescence signal yield of conventional fluorescence labels of up to a factor of 25 for the same number of applied excitation pulses.⁴ However, implementing T-Rex with decreasing repetition rates is not optimal because of the increase in acquisition time. An alternative is to move a scanning beam so quickly that a given coordinate in the sample is illuminated over a very short period of time only.^{4,9–12} Because such rapid scanning may not always be realizable, a good compromise is to employ only a few illumination pulses on the same sample spot. This scheme can be mimicked by implementing pulse sequences consisting of short illumination periods that are accompanied by longer idle times.

Hence, we investigated different excitation patterns of bunched pulsed excitation with the aim to quantify the potential increase in signal and photostability. Taking the organic dye Atto532 and the green fluorescent protein (GFP) as examples, we explore to what extent bunches of high repetition pulses followed by an idle time for dark state relaxation ($\sim\mu\text{s}$) cut down illumination times while still operating in the T-Rex regime.

In brief, we found a good compromise between the numbers of pulses applicable in these bunches (and thus of the acceleration in scanning or recording time), and the achievable improvement in fluorescence signal. For a given number of pulses, the improvement was the same irrespective of whether the pulses were applied in bunches or evenly spread out over the acquisition time. This study corroborates the finding that the photobleaching of these exemplary fluorophores is strongly influenced by the population and subsequent excitation of a long-lived dark state. Our findings are relevant for a whole range of bioanalytical fluorescence techniques that are challenged by photobleaching.

Experimental

A monolayer of the organic dye Atto532 (fluorescence excitation and emission maxima at 532 and 553 nm, respectively; Atto-Tec, Siegen, Germany) was realized by coupling the dye to a silanised cover glass *via* its NHS linker. Purified green fluorescent protein (GFP) was analyzed in a ~ 500 nm thick layer adsorbed on a cover glass. The layers were mounted by Mowiol (13% w/v Mowiol 4–88 (Fluka, Buchs, Switzerland), 33% w/v glycerol, 33% v/v double-distilled water, 37% v/v tris-buffer pH 8, 0.1% w/v Dabco (1,4-diazabicyclo[2.2.2]octane (Fluka))). In the experiments comprising the triplet quencher, 2 mM 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany) was added into the mounting media Mowiol before coverage of the samples. For further details on GFP

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purification as well as on the preparation of the samples see ref. 4 and 13.

The pulsed T-Rex experiments were performed on a standard epi-illuminated confocal microscope. Briefly, we excited the fluorophores with a pulsed 470 nm laser diode (LDH-P-C-470, Picoquant GmbH, Berlin, Germany) featuring a pulse width of ~ 100 ps. The timing of the pulses was realized by a home-build external electronic trigger device. The microscope featured a piezo-controlled scanning stage (NanoMax, Thorlabs) and an oil immersion objective (HCX PL APO 100 \times /1.4, Leica, Mannheim, Germany) producing nearly diffraction-limited excitation spots or intensity point-spread-functions (PSF). The fluorescence emission was collected by the same objective lens and projected onto a counting avalanche photodetector (SPCM-AQR-13-FC, Perkin Elmer Optoelectronics, Fremont, CA) with an aperture size corresponding to 0.8 times the magnified Airy disk of the fluorescence spot. The detection events were further processed by a PC card (SPC 730, Becker & Hickl GmbH, Berlin, Germany), enabling the observation of the fluorescence count rate within varying observation time windows. The excitation PSF was probed by a gold bead of 80 nm diameter (En.GC80, BBInternational, Cardiff, UK) on a nonconfocal detector (MP 963 Photon Counting Module, Perkin Elmer). The full width at half maximum (FWHM) of the PSF in the focal plane and the power P measured at the sample entered the calculation of the applied pulse peak intensities $I_p = P/[\pi(0.5 \text{ FWHM})^2(\tau_p/f)]$ with pulse length τ_p and time-averaged laser repetition rate f .

Results and discussion

Maximizing the sensitivity of fluorescence microscopy often requires maximizing the fluorescence rate, *i.e.* the fluorescence emission within a certain time span, which is usually approached by applying large excitation intensities of laser light. However, the photophysical and -chemical characteristics of conventional fluorophores introduce an upper limit to this approach. Fig. 1 recalls the main reasons of this limitation: population of a relatively long-lived dark state and light-induced photobleaching.^{3,4,8,14} Several intra-molecular pathways may follow the excitation of the fluorophore from its electronic ground S_0 to its first excited

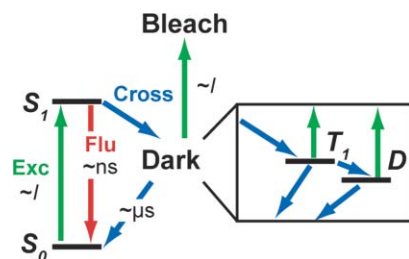


Fig. 1 Population of and photobleaching from long-lived dark states introduce saturation of the fluorescence emission of typical fluorophores, limiting their total fluorescence yield. Repetitive excitation (Exc) of the singlet ground state S_0 to the first excited singlet state S_1 by light of intensity I elicits emission of a fluorescence photon (Flu) within a few nanoseconds, or crossing to a dark state with lifetimes of $\sim \mu s$ in which the fluorophore is vulnerable to further optical excitation ($\sim I$) to higher energy levels and hence to bleaching (Bleach). Examples of the dark state are the lowest excited triplet state T_1 or other dark states D populated *via* T_1 (inset).

electronic singlet state S_1 , of which fast ($\sim ns$) $S_1 \rightarrow S_0$ de-excitation by fluorescence emission is the desired one. Crossing to a dark state such as the triplet state is unwanted. Dark states are usually relatively long-lived with lifetimes in the range of μs for triplet states in aqueous environment.¹⁵ Consequently, they act as a trap impeding the fluorophore's return to S_0 and dislodging it from further excitation-emission cycles. Thus, trapping in the dark state sets an upper limit of the fluorescence rate. Moreover, the dark state's longevity promotes photochemical reactions and subsequent photobleaching *via* absorption to dark states of higher energy, leading to an irreversible loss of fluorescence emissivity.⁶⁻⁸

These multi-step photobleaching reactions are especially pronounced for fluorescence excitation modes applying high-intensity continuous wave (CW) or high (40–80 MHz) repetition laser light. They are also inherent to excitation modalities requiring high light levels such as two- or multi-photon absorption¹⁶⁻¹⁸ or stimulated emission depletion (STED) microscopy.³ Reducing the repetition rate of pulsed excitation is a straightforward way to minimize dark state population and photobleaching and to maximize the achievable fluorescence signal because the prolonged lag time between laser pulses allows for dark state relaxation.^{3,4} In a number of cases, the implementation of this triplet- (or dark-state) relaxation (T- or D-Rex) excitation mode requires laser pulse picking of the initially 80 MHz pulse trains, which leads to increased acquisition times. It is therefore important to explore excitation modes that maintain the T-Rex advantage but accelerate signal acquisition and are applicable with laser scanning microscopes (LSM). Bunched pulsed excitation may serve as an intermediate between CW or continuous high-repetitive pulsed excitation and the low repetition rate T-Rex excitation mode and, at the same time, mimic the fast scanning process of an LSM in which short illumination periods or bunches of a few high-repetitive pulses are separated by a longer time of absent illumination.

We studied the influence of pulse bunching on the increase in photostability and fluorescence signal by implementing trains of 5, 10, and 21 excitation pulses having 25 ns time intervals between succeeding pulses. The time difference between the first pulses of each bunch were $\sim 2.5 \mu s$ which is usually long enough to allow for a pronounced T- or D-relaxation effect (Fig. 2B). Such bunching mimics dwell times per fluorophore of ~ 100 –500 ns in laser scanning microscopy. The bunched modes are benchmarked against a continuous 40 MHz and a 400 kHz pulse train; the latter fulfils T-Rex conditions for the fluorophores used.^{3,4}

Fig. 2A depicts the results on a thin layer of GFP spread on cover glass. Single spots of the layer were exposed to the different illumination schemes. Importantly, the total number of 5×10^5 pulses was kept constant by adjusting the total exposure time accordingly. The photobleaching was visualized and quantified by subsequent imaging of the exposed and hence photobleached spots. The revival of photobleached areas by molecular diffusion was precluded by covering the layer with the mounting medium Mowiol. The different illumination schemes were implemented with four different peak intensities I_p of the pulsed 470 nm excitation light showing increased photobleaching with increasing I_p .

Importantly, photobleaching decreases with the number of pulses within one bunch. This is evidenced for the different

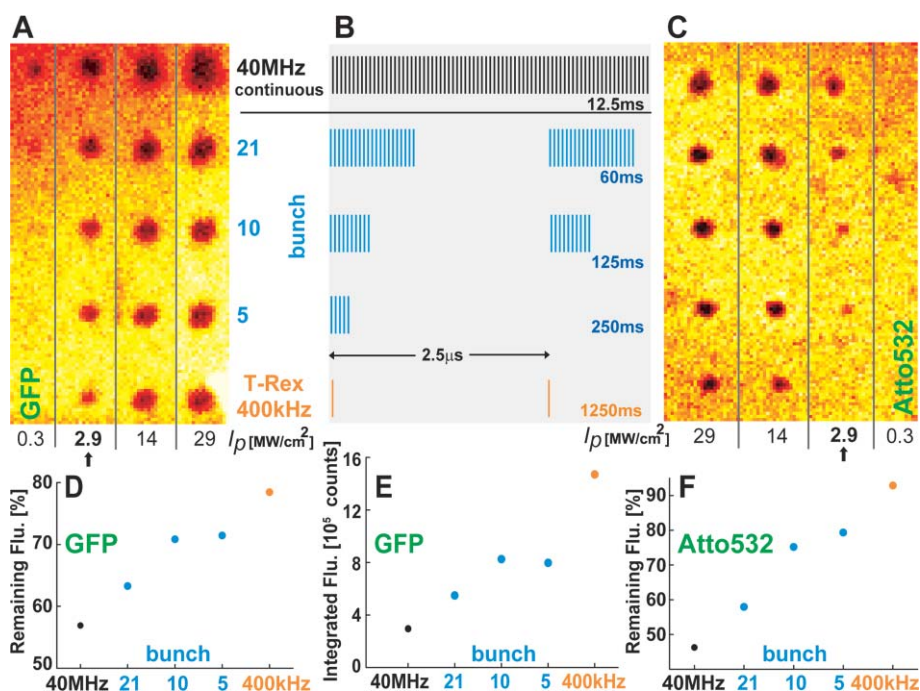


Fig. 2 Photobleaching and fluorescence signal following bunched T-Rex excitation on fluorophore layers consisting of GFP and Atto532. Illumination with a total number of 5×10^5 pulses leaves dark spots (A, C) with different levels of remaining fluorescence as given in (D, F) depending on the pulse intensity I_p and the pulse timing of illumination. Bunched excitation that is repeated in time intervals of $2.5 \mu\text{s}$ with 21, 10 and 5 pulses within each bunch were compared to a continuous 40 MHz and a 400 kHz T-Rex pulse train. The timelines of the different pulse trains are plotted schematically giving different total acquisition times (B). The time difference between the individual pulses in the bunches was $1/(40 \text{ MHz}) = 25 \text{ ns}$ with dark periods filling up the time interval of $2.5 \mu\text{s}$. For GFP, the integrated fluorescence signal of the different illumination modes is given as well (E). The levels of remaining fluorescence and integrated fluorescence are plotted for $I_p = 2.9 \text{ MW cm}^{-2}$.

excitation schemes by the measured residual fluorescence after illumination with the 5×10^5 pulses of $I_p = 2.9 \text{ MW cm}^{-2}$ (Fig. 2D). While the pure T-Rex scheme (400 kHz) leaves 80% of the fluorescence in place, bunching into 5 or even 10 pulses yields almost the same level ($\sim 70\%$) of remaining fluorescence. A further increase of the number of pulses in a bunch reduces the remaining fluorescence further. The largest bleaching is found for the continuous 40 MHz pulse train, leaving only $\sim 55\%$ of the initial fluorescence signal. The increase in photostability with bunched pulsed illumination is accompanied by an increase in total fluorescence signal integrated over the total illumination time (Fig. 2E). For $I_p = 2.9 \text{ MW cm}^{-2}$, bunching into 5 to 10 pulses yields a ~ 2.5 -fold increased total signal as compared to continuous 40 MHz excitation; this value is only exceeded by the ~ 5 -fold increase found for the 400 kHz T-Rex illumination scheme. Similar factors were obtained for larger intensities I_p , indicating that we have reached excitation saturation.⁴ Measurements on a layer of the organic dye Atto532 outline the same characteristics (Fig. 2C,F) indicating the generality of the observed phenomenon.

The time interval of $\sim 2.5 \mu\text{s}$ between succeeding bunches obviously allows for the relaxation of the $\sim 1 \mu\text{s}$ long-lived triplet state. However, the probability of triplet population increases with the number of pulses within a bunch causing the photostability and total fluorescence yield to degrade with the number of pulses per bunch. The similar levels of photobleaching and fluorescence signal obtained for the 5- and 10-bunch illumination scheme probably result from a less accurate timing adjustment in the case of the 5-bunch experiment. In any case, recording in bunches

provides substantial improvements over regular high repetition rate excitation, although it does not reach all the advantages of the ideal T-Rex scheme. Compared to the latter, bunched excitation accelerates the acquisition times according to the number of pulses used in the bunch, *i.e.* 10-fold when applying a train of 10 pulses. Therefore, the 1.3-fold increase in photostability and the 2.5-fold increase in fluorescence signal (over the 40 MHz case) realized with these bunches, which is lower than the corresponding factors of 1.5 and 5 obtained with the nearly ideal T-Rex modality, has to be weighed against the 10-fold cut down of the T-Rex recording time from 1.25 s to 0.125 s.

Further evidence for the advantages of the bunched pulses is provided by the comparison with a continuous low repetition rate mode featuring the same total illumination time. To this end, 5- and 10-pulse bunching is benchmarked against continuous illumination with repetition rates of 2 MHz and 4 MHz, respectively (Fig. 3A). Instead of being penned up in bunches and leaving an idle time of $2.5 \mu\text{s}$ between the first pulses of each bunch, the same total number of pulses are now evenly distributed in time. Fig. 3B shows the bleaching pattern of the Atto532 layer illuminated with 5×10^5 pulses of four different peak intensities I_p . It directly compares the 5-pulse bunching scheme against a continuous 2 MHz and the 10-pulse bunching scheme against a continuous 4 MHz excitation pattern. Again, the largest photobleaching is generated by illumination with a continuous repetition rate of 40 MHz, while no difference in bleaching is observed between the various illumination patterns. Similarly, we do not observe a difference in the total fluorescence signal detected

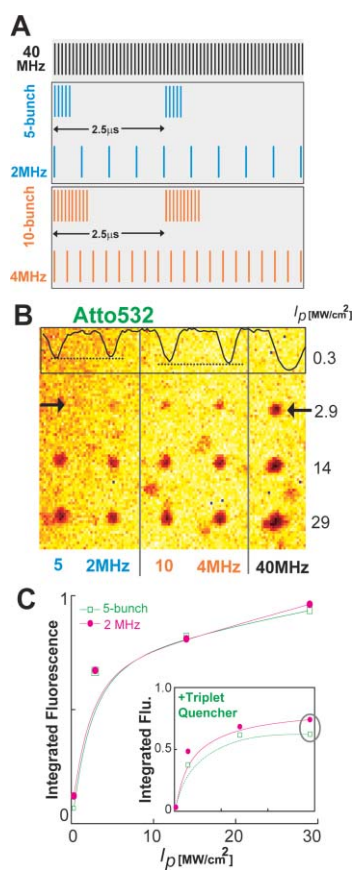


Fig. 3 Comparison of pulsed fluorescence excitation in bunches and at constant repetition on a dye layer of Atto532. Various trains consisting of bunches of 10 and 5 excitation pulses, arriving in time intervals of 2.5 μ s were compared with a continuous 40 MHz pulse train. The timelines of the different pulse trains are schematically plotted in (A). The 10 and 5 pulses were either applied with a repetition rate of 40 MHz with dark periods filling up the remainder of the 2.5 μ s, or evenly distributed in time with repetition rates of 4 and 2 MHz, respectively. Illumination with a total number of 5×10^5 pulses and thus with total acquisition times of 250 ms (5-bunch or 2 MHz) and 125 ms (10-bunch or 4 MHz) leaves differently photobleached dark spots (B) with levels of remaining fluorescence (inset B) and integrated fluorescence signal (C) depending on the pulse intensity I_p and the pulse timing of illumination. The levels of remaining fluorescence (profile across the photobleached dye layer) and integrated fluorescence are plotted for $I_p = 2.9 \text{ MW cm}^{-2}$ (arrow). Under these conditions, the same number of pulses leads to the same levels of photobleaching and to a similar amount of integrated fluorescence signal at different I_p (C), which changes upon addition of a triplet quencher (inset C), favoring excitation with a uniform repetition of pulses (circle). The lines in (C) are drawn for guidance.

during illumination between the bunched and non-bunched modes (Fig. 3C).

The time distance between succeeding pulses of 0.5 and 0.25 μ s of the continuous 2 and 4 MHz illumination mode, respectively, is smaller than the triplet lifetime of $\sim 1 \mu$ s in the samples investigated.⁴ Only dark periods exceeding the average time needed for triplet relaxation render ideal T-Rex illumination.⁴ In our case, nearly ideal T-Rex illumination is attained only with interpulse time differences of $>2.5 \mu$ s, *i.e.* pulse repetition rates $<400 \text{ kHz}$. Hence, for the not yet ideal T-Rex conditions of continuous 2 or 4 MHz excitation, the triplet population basically only depends on

the number of pulses distributed during the time interval of 2.5 μ s, but not on the timing of the pulses. In other words, the bunched illumination mode is not expected to be disadvantageous. We confirmed these observations by calculations based on common dye parameters and a conventional electronic state model¹⁸ (data not shown).

We provided further evidence for this characteristic by adding 2 mM of the triplet quencher 2-mercaptoethanol in order to reduce the triplet lifetime of our samples.¹⁹ The presence of the triplet quencher shortens the triplet lifetime far below 1 μ s, shifting the ideal T-Rex condition to repetition rates $>400 \text{ kHz}$, *i.e.* to $\sim 1\text{--}2 \text{ MHz}$. The inset to Fig. 3C shows the fluorescence signal of the Atto532 layer in the presence of the triplet quencher again obtained for a total of 5×10^5 pulses, for 5 pulses either bunched or evenly distributed over the duty cycle of 2.5 μ s. As before for the 400 kHz T-Rex mode (Fig. 2), the (in this case ideal) 2 MHz T-Rex illumination is now favored over pulse bunching, resulting in about 10% less signal of the bunched illumination as compared to its continuous counterpart. This difference can also be theoretically explained for well determined kinetic parameters of rhodamine dyes,¹⁸ specifically by a ~ 10 -fold shorter triplet lifetime.

This observation also highlights the dependence of our approach on environmental conditions. Since the lifetime of the triplet state may change with experimental conditions such as additives, concentration of molecular oxygen, or temperature, adjusting the timing of the experiment is required. Yet, the triplet lifetime is usually prolonged to $>1 \mu$ s in biological samples or other mounting media, thus shifting the ideal T-Rex illumination regime to repetition rates $<400 \text{ kHz}$. Under these circumstances, the disadvantages of applying pulse bunching over low repetition rate illumination are not given when applying time differences between bunches in the order of 2.5 μ s.

Since the repetition rate of a laser system is usually fixed, bunched excitation can be realized using acousto-optical-modulators as fast shutters or simply with any fast laser scanning microscope that allows reducing the pixel dwell times to the bunch period, *i.e.* to $\sim 100\text{--}200 \text{ ns}$. In this case, the excitation need not be pulsed. With pulse widths in the range of $\sim 100 \text{ ps}$ as applied here, there is hardly any difference between CW and (quasi-CW) pulsed excitation, if the average power is kept the same.^{4,18,20} Common laser scanning microscopes equipped with a fast scanning device and simple CW excitation sources can thus acquire the advantages achieved with bunched illumination.^{4,9-12} Hence, fast scanning allows application of much larger CW laser powers and thus yields much larger signal levels than single-point CW excitation experiments, since the optimal laser power is less limited by dark state populations. Furthermore, since the T-Rex illumination scheme also applies to two- or multi-photon excitation⁴ or high-resolution STED microscopy,³ the concept of bunched illumination or fast scanning also applies to these techniques.

Illumination in short bunches or with fast scanning times, as presented here, effectively bridges the gap between the regime of conventional high repetition rate or CW and ideal low repetition rate T-Rex excitation (Fig. 4). Therefore, the experimentally accessible freedom for choosing between a large total signal and short acquisition time is thoroughly expanded. Since either the needed signal or the maximum recording time provides the stronger condition of the actual experiment, the less constrained

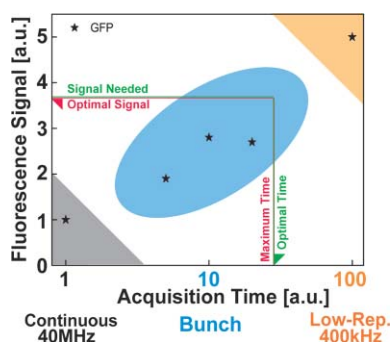


Fig. 4 Balancing between large fluorescence signal and short acquisition time for fluorescence data recording applying the different modes of excitation: continuous (fast 40–80 MHz pulsed or CW excitation), excitation in bunches with an increasing number of pulses within a bunch and excitation with continuous low (400 kHz) repetition rate. Experimental data recorded for the GFP dye layer are given as black stars. CW or 40 MHz pulsed excitation realizes short acquisition times but also low levels of signal due to pronounced dark state populations (grey area). Excitation with low repetition rates renders large signal levels at the expense of long acquisition times (orange area). This gap can be bridged by applying bunched excitation (blue area) providing a good compromise between fluorescence signal and acquisition time.

variable can be optimized for choosing the suitable excitation scheme.

Conclusion

Our study demonstrates that within a certain range fast scanning or bunched excitation provides an effective way of improving the photostability and fluorescence signal brought about by the T-Rex excitation scheme. Limiting the excitation to repetitive short periods of ~100–200 ns followed by breaks that are long enough for dark (triplet) state relaxation reduces the recording time while still operating close to T-Rex conditions. This observation holds true both for Atto532, a prominent member of the rhodamine dye family, and GFP, the archetype of fluorescent proteins. The verification of this concept with these fluorophores indicates the generality of the concept. It is extendable to both CW and multi-photon excitation, as well as to STED imaging. As a result, the bunched excitation scheme provides new degrees of freedom for balancing between signal maximization and imaging speed, thus complementing the existing modes of fluorescence excitation.

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References

- 1 R. Y. Tsien, Imaging imaging's future, *Nat. Cell Biol.*, 2003, SS16–SS21.
- 2 J. B. Pawley, (ed.), *Handbook of biological confocal microscopy*, Springer, New York, 2nd edn., 2006.
- 3 G. Donnert, J. Keller, R. Medda, M. A. Andrei, S. O. Rizzoli, R. Lührmann, R. Jahn, C. Eggeling and S. W. Hell, Macromolecular-scale resolution in biological fluorescence microscopy, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 11440–11445.
- 4 G. Donnert, C. Eggeling and S. W. Hell, Major signal increase in fluorescence microscopy through dark-state relaxation, *Nat. Meth.*, 2007, **4**, 81–86.
- 5 M. Kasha, Paths of molecular excitation, *Radiat. Res.*, 1960, **2**, 243–275.
- 6 M. Anbar and E. Hart, The reactivity of aromatic compounds towards hydrated electrons, *J. Am. Chem. Soc.*, 1964, **86**, 5633–5637.
- 7 E. V. Khoroshilova and D. N. Nikogosyan, Photochemistry of uridine on high intensity laser UV irradiation, *J. Photochem. Photobiol. B*, 1990, **5**, 413–427.
- 8 C. Eggeling, J. Widengren, R. Rigler and C. A. M. Seidel, Photobleaching of fluorescent dyes under conditions used for single-molecule detection: Evidence of two-step photolysis, *Anal. Chem.*, 1998, **70**, 2651–2659.
- 9 W. W. Webb, K. S. Wells, D. R. Sandison, and J. Strickler, Criteria for quantitative dynamical confocal fluorescence imaging, in *Optical Microscopy for Biology*, ed. B. Herman, and K. Jacobson, Wiley, New York, 1990, pp. 73–108.
- 10 J.-A. Conchello and J. W. Lichtman, Optical sectioning microscopy, *Nat. Meth.*, 2005, **2**, 920–931.
- 11 R. Y. Tsien, L. Ernst, and A. Waggoner, Fluorophores for Confocal Microscopy: Photophysics and Photochemistry, in *Handbook of biological confocal microscopy*, ed. J. B. Pawley, Springer, New York, 2006, pp. 338–352.
- 12 R. T. Borlinghaus, High Speed Scanning Has the Potential to Increase Fluorescence Yield and to Reduce Photobleaching, *Micr. Res. Tech.*, 2006, **69**, 689–692.
- 13 J. Fölling, M. Bossi, H. Bock, R. Medda, C. A. Wurm, B. Hein, S. Jakobs, C. Eggeling and S. W. Hell, Fluorescence nanoscopy by ground-state depletion and single-molecule return, *Nat. Meth.*, 2008, **5**, 943–945.
- 14 C. Eggeling, J. Widengren, R. Rigler, and C. A. M. Seidel, Photostabilities of fluorescent dyes for single-molecule spectroscopy: Mechanisms and experimental methods for estimating photobleaching in aqueous solution, in *Applied fluorescence in chemistry, biology and medicine*, ed. W. Rettig, B. Strehmel, M. Schrader, and H. Seifert, Springer, Berlin, 1999, pp. 193–240.
- 15 J. Widengren, Ü. Mets and R. Rigler, Fluorescence correlation spectroscopy of triplet states in solution: A theoretical and experimental study, *J. Phys. Chem.*, 1995, **99**, 13368–13379.
- 16 G. H. Patterson and D. W. Piston, Photobleaching in two-photon excitation microscopy, *Biophys. J.*, 2000, **78**, 2159–2162.
- 17 P. S. Dittrich and P. Schwill, Photobleaching and stabilization of fluorophores used for single-molecule analysis with one- and two-photon excitation, *Appl. Phys. B*, 2001, **73**, 829–837.
- 18 C. Eggeling, A. Volkmer and C. A. M. Seidel, Molecular Photobleaching Kinetics of Rhodamine 6G by One- and Two-Photon Induced Confocal Fluorescence Microscopy, *ChemPhysChem*, 2005, **6**, 791–804.
- 19 J. Widengren, A. Chmyrov, C. Eggeling, P.-A. Löfdahl and C. A. M. Seidel, Strategies to Improve Photostabilities in Ultrasensitive Fluorescence Spectroscopy, *J. Phys. Chem. A*, 2007, **111**, 429–444.
- 20 I. Gregor, D. Patra and J. Enderlein, Optical Saturation in Fluorescence Correlation Spectroscopy under Continuous-Wave and Pulsed Excitation, *ChemPhysChem*, 2004, **5**, 1–7.