EGFP and DsRed expressing cultures of *Escherichia coli* imaged by confocal, two-photon and fluorescence lifetime microscopy

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Abstract The green fluorescent protein (GFP) has become an invaluable marker for monitoring protein localization and gene expression in vivo. Recently a new red fluorescent protein (drFP583 or DsRed), isolated from tropical corals, has been described [Matz, M.V. et al. (1999) Nature Biotech. 17, 969–973]. With emission maxima at 509 and 583 nm respectively, EGFP and DsRed are suited for almost crossover free dual color labeling upon simultaneous excitation. We imaged mixed populations of *Escherichia coli* expressing either EGFP or DsRed by one-photon confocal and by two-photon microscopy. Both excitation modes proved to be suitable for imaging cells expressing either of the fluorescent proteins. DsRed had an extended maturation time and *E. coli* expressing this fluorescent protein were significantly smaller than those expressing EGFP. In aging bacterial cultures DsRed appeared to aggregate within the cells, accompanied by a strong reduction in its fluorescence lifetime as determined by fluorescence lifetime imaging microscopy.

Key words: Green fluorescent protein; Red fluorescent protein; Confocal; Two-photon; Fluorescence lifetime microscopy; *Escherichia coli*

1. Introduction

The exploitation of the green fluorescent protein (GFP) as an in vivo reporter for subcellular localization and protein expression has revolutionized cell biology over the last decade [2,3]. GFP folds autocatalytically without the need for external substrates or co-factors. It has been fused to a variety of proteins in both eukaryotic and bacterial systems. The localization and spatial dynamics of such fusion proteins can be monitored non-invasively by fluorescence microscopy in living cells. Mutagenesis of the original GFP from the jellyfish *Aequorea* has improved aspects such as photostability, quantum efficiency, codon usage and thermosensitivity, and yielded variants fluorescent in hues ranging from blue to yellow. Multilabeling experiments with different GFP-variants have been successfully performed, although the discrimination of the different GFP-isoforms was partly complicated by their spectral overlap [4,5]. Due to their overlapping emission spectra, simultaneous excitation of two different GFP-variants, which is desirable for time-lapse experiments, required post-experimental mathematical crossover correction [4,6].

An alternative might be provided by a new addition to the set of fluorescent proteins, a red fluorescent GFP homologue (drFP583; sold as DsRed by Clontech) that has been recently isolated from the Indo Pacific reef coral *Discosoma* sp. Its emission spectrum has a maximum at 583 nm, clearly separated from the 509 nm emission peak of the green EGFP [7]. Therefore, a combination of EGFP and DsRed appears to be promising for double labeling studies with negligible crosstalk.

Genetically the Gram-negative bacterium *Escherichia coli* is one of the best-known organisms. Despite the vast knowledge on bacterial gene regulation comparatively little is known about *E. coli* cell biology. Whereas in eukaryotic cell biology GFP is one of the most versatile tools, it has been adapted only recently for use in bacterial cells [8]. To investigate the feasibility of using EGFP and DsRed for double-labeling in bacteria we expressed both fluorescent proteins separately in *E. coli*. We analyzed qualitatively the capabilities of confocal [9] and two-photon microscopy [10,11] to discriminate cells expressing EGFP or DsRed upon simultaneous excitation. Two-photon laser scanning microscopy (TPLSM) has been successfully used for long term observations of live specimen [12] at a resolution that is close to that of a confocal laser scanning microscopy (CLSM) [13]. Prominent advantages of two-photon over single-photon excitation include the ability to observe layers deep down in scattering tissue, reduced total photobleaching and longer viability for specimens thicker than about 20 μm [12]. A confocal pinhole is not required for optical sectioning and one is usually able to excite two or more fluorophores with the same wavelength [10]. When imaging small cells like *E. coli* only the latter advantages apply. However, we expanded our study to two-photon excitation in order to determine if this excitation mode is suitable for DsRed imaging.

The standard intensity and emission wavelengths contrast modes have lately been complemented by fluorescence lifetime imaging microscopy (FLIM) [14,15]. FLIM is receiving increased interest [16–18] because the decay rate of specific fluorophores is indicative of the chemical microenvironment of a dye. FLIM can be used to visualize the spatial distribution of ion concentrations, pH or the binding to macromolecules. In particular, the decrease of the donor fluorescence lifetime in a pair of fluorophores undergoing fluorescence resonance energy transfer (FRET) [19] is a stringent criterion for FRET. Frequency-domain FLIM has successfully been used to separate co-expressed spectrally similar GFP variants in mamma-

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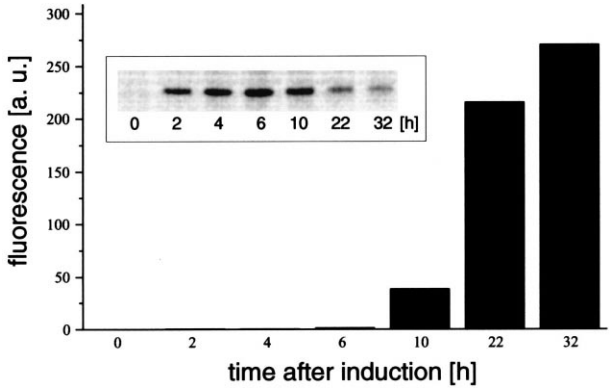

Fig. 1. Overall fluorescence of an *E. coli* culture following induction of DsRed expression. Excitation at 550 nm, detected fluorescence between 560 and 700 nm. Data are normalized to an absorbance at 600 nm to account for cell growth and are normalized to 1 for the fluorescence of the uninduced sample. Inset: section of Coomassie-stained gel demonstrating DsRed expression. Equal amounts of total protein extract have been applied on the gel.

lian cells [20]. Here, we used time-correlated single-photon counting (TCSPC) to discriminate *E. coli* cells expressing either EGFP or DsRed.

2. Materials and methods

2.1. Bacterial strains and media

The DNA sequence encoding EGFP, a gift from Dr. David Piston (Vanderbilt University, Nashville, TN, USA), was cloned into the vector pRSETa (Invitrogen). The DsRed coding sequence was excised from the pDsRed1-N1 plasmid (Clontech) and inserted into the pRSETa vector. These plasmids encode expression of the fluorescent proteins with an additional six histidines at the amino terminus, under control of the T7 promoter inducible by isopropyl-β-D-thiogalactoside (IPTG). *E. coli* BL21 (DE3) cells were transformed with the plasmids according to standard protocols, and grown in LB medium at 37°C using ampicillin for selection. Protein expression was induced by 1 mM IPTG.

2.2. Sample preparation

Coverslips were coated with poly-L-lysine (Sigma, St. Louis, MO, USA) to facilitate attachment of the cells on the glass. *E. coli* resuspended in LB-medium were placed between slide and coverslip and sealed with nail-polish.

2.3. Single-photon confocal microscopy

A standard beam scanning microscope (Leica TCS SP, Leica Lasertechnik, Heidelberg, Germany) with adjustable bandwidths of the detected fluorescence wavelengths was used. We employed a 1.4 numerical aperture oil immersion lens (Leica 100×, Planapo, Wetzlar, Germany). Simultaneous excitation was achieved by the 488 and 568 nm lines from an ArKr-laser. EGFP fluorescence was detected from 490 to 540 nm and concurrently the DsRed emission from 620 to 670 nm. Both photomultipliers were adjusted to the same sensitivity.

2.4. Two-photon microscopy

For two-photon imaging we used a converted commercial beamscanning microscope (Leica TCS NT) employing a mode-locked Ti:-sapphire Laser (Coherent Mira 900) pumped by an 8 W argon-ion laser (Coherent Innova 300), tuned to 925 nm. To separate the infrared excitation from the fluorescence we used a 660 nm long-pass dichroic mirror mounted at a 45° angle to the beam. After deflection the fluorescence light was further split by a 540 nm short-pass dichroic mirror and directed to two photomultipliers (PM; R6357, R6358, Hamamatsu, Japan). The red sensitive PM R6357 was utilized for DsRed detection. To block residual EGFP fluorescence a 590 nm longpass filter (3 mm OG590, Schott) was placed in front of the photomultiplier opening. To further prevent residual laser light from being detected, the backpropagating light passed IR-blocking filters (3 mm BG39, Schott). No pinholes were used; the detection was performed in a non-descanned mode. The PM for DsRed detection was set to a higher voltage than the PM for EGFP detection. For two-photon microscopy with 1064 nm excitation we used the same setup as for FLIM (see below), but replaced the laser by a Nd:Glass laser (Time Bandwidth Products, Zürich, Switzerland). The laser delivered ~200 fs pulses at a repetition rate of ~120 MHz. The power of the excitation light in the focus was about 1 mW.

2.5. FLIM

We utilized a stage-scanning two-photon microscope [21] with a large (200 μm) pinhole to keep out scattered light. Excitation was achieved by a pumped Ti:sapphire laser tuned to 950 nm. For detection we employed a counting avalanche photodiode (Perkin-Elmer, SPCM 500, Vaudreuil, Canada) in combination with a commercial time-correlated single-photon counting (TCSPC) module (B and H SPC535, Berlin, Germany). We measured an instrument response function of 420 ps (full-width-half-maximum) for our system and measured histograms of 1024 time bins with an individual width of 25 ps. The electrical time resolution of the module is approximately 7 ps [22]. A short pass filter (3 mm of KG3, Schott) in combination with a dichroic bandpass (HQ680SP, Chroma) was used to block laser light from the detector.

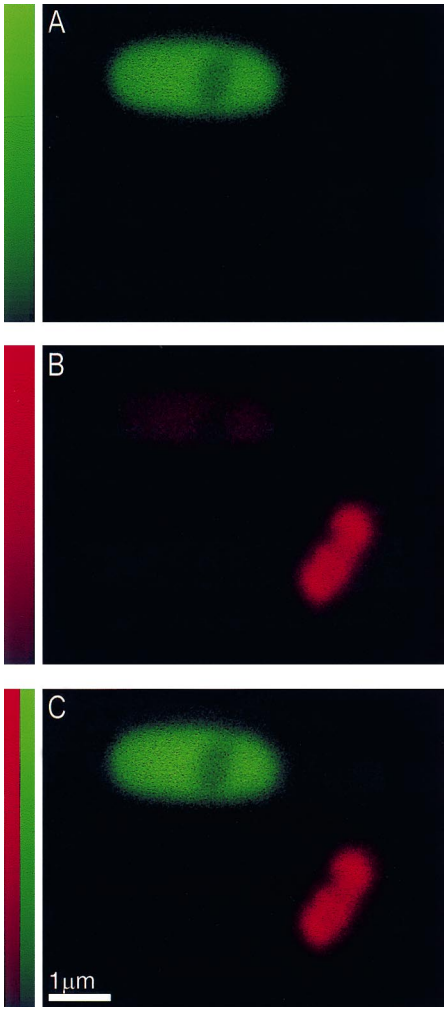

Fig. 2. *E. coli* expressing EGFP or DsRed imaged with CLSM. Simultaneous excitation with 488 nm and 568 nm. A: Emission recorded at 490–540 nm (EGFP). B: Emission recorded at 620–670 nm (DsRed). C: Overlay of both pictures. The images are the average of four single optical slices with a resolution of 512×410 pixels taken at a pixel dwell time of 1 μs.

3. Results and discussion

3.1. Expression of EGFP and DsRed in E. coli

Expression of the fluorescent proteins by *E. coli* transformed with plasmids encoding EGFP and DsRed was induced by addition of 1 mM IPTG. Whereas EGFP fluorescence could clearly be seen in the cells 4 h after induction, DsRed required ~20 h after induction for efficient microscopic visualization. This delay was not due to inefficient expression of the DsRed protein, since the protein was detectable in high quantities already 2 h after induction (see inset Fig. 1). Measurements of the overall fluorescence of DsRed expressing cultures revealed an increase in fluorescence beginning ~10 h after induction (Fig. 1). The discrepancy between the appearance of DsRed protein and DsRed fluorescence indicates an extended maturation time of the protein. Furthermore, under the conditions employed, cells expressing DsRed were in general markedly smaller than cells expressing EGFP or untransformed bacteria (Fig. 2). The apparent influence of DsRed on cell size might indicate a toxic effect of the expressed recombinant protein, and deserves a more thorough analysis. In cells expressing EGFP the fluorescence was almost always evenly distributed throughout the cytoplasm. Cultures with EGFP could be stored over weeks at 4°C without changing the apparent intracellular distribution of the fluorescent protein. In contrast, we found DsRed fluorescence in a varying fraction of cells to be localized. This effect was strongly enhanced when the cell culture was stored over several days, potentially indicating precipitation or aggregation of DsRed within the cytoplasm.

3.2. Single-photon confocal microscopy

Suspensions of EGFP or DsRed expressing cells showed emission spectra (data not shown) similar to those of purified proteins with emission peaks at 509 and 583 nm, respectively [1]. We note, however, that the maturation of the DsRed protein was accompanied by a shift in its emission spectrum. To maximize fluorescence yield we choose 488 and 568 nm for excitation, which are almost in the maximum of the excitation spectra of the respective proteins. In order to minimize crosstalk the detected fluorescence was selected to range from 490 to 540 nm for EGFP, covering the maximal EGFP emission, and from 620 to 670 nm for DsRed. The collected signal for the red channel did not include the emission maximum of DsRed in order to suppress residual EGFP fluorescence. It is also noteworthy that the PM quantum efficiency is lowered to about 70% for the red channel. Nonetheless, sufficient DsRed signal could be collected. Obviously, for samples with weak DsRed fluorescence higher signals could be gained by adjusting the collected fluorescence to the DsRed emission maximum, although this measure would increase crossover. Under our experimental conditions using simultaneous excitation and detection almost no crossover from DsRed was detectable in the EGFP channel (Fig. 2a), while the crossover from EGFP in the DsRed channel was consistently ≤5% (Fig. 2b). Therefore, cells expressing either EGFP or DsRed could be separated easily and cleanly without any image processing (Fig. 2c). This is an advantage over the frequently used CFP/YFP (cyan/yellow) combination, which inevitably requires, upon simultaneous excitation, image processing for quantitative separation [4,6].

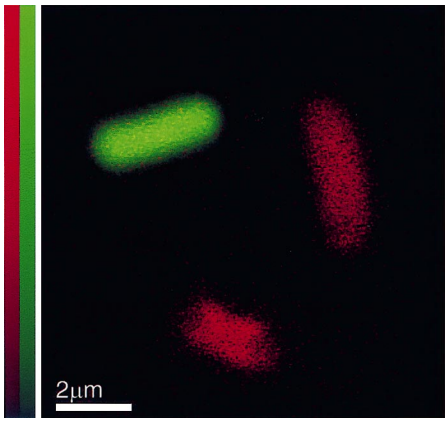

Fig. 3. *E. coli* expressing EGFP or DsRed imaged simultaneously with a two-photon laser scanning microscope. Excitation wavelength 925 nm. Overlay of both detection channels (for details see text). Image resolution was 512×512 pixels, dwell time and averaging as in Fig. 2.

3.3. Two-photon microscopy

The mode-locked Ti:sapphire laser was tuned to 925 nm and used to simultaneously excite EGFP and DsRed expressing cells (Fig. 3). Excitation of both fluorophores in the infrared avoids chromatic aberrations, which is advantageous when both proteins are co-localized in small structures. At 925 nm, EGFP expressing cells were much brighter than DsRed cells, requiring careful adjustment of laser power and photomultiplier settings. The gross difference in brightness is not surprising, since this wavelength was probably well outside the maximum for two-photon excitation of DsRed. The DsRed fluorescence was further curtailed by the reduced sensitivity of the photomultiplier for longer wavelengths and the filter settings used. With our setup no DsRed fluorescence was bleeding into the 'green' channel and the crossover from EGFP in the DsRed channel was ≤15%. Although the 925 nm used in this study is sufficient to visualize *E. coli* overexpressing DsRed it is questionable whether this wavelength is suited for imaging cells expressing less abundant protein levels.

In order to approach more optimal excitation conditions for DsRed we explored two-photon excitation by a Nd:Glass laser emitting at 1064 nm (Fig. 4). The fluorescence of DsRed expressing cells appeared bright and photostable when excited at this wavelength. Since EGFP is almost not excitable at 1064 nm (data not shown), this wavelength is not suitable for imaging EGFP/DsRed double-labeled cells. Assuming that the two-photon excitation spectrum can be inferred from the doubled excitation wavelengths of its one-photon

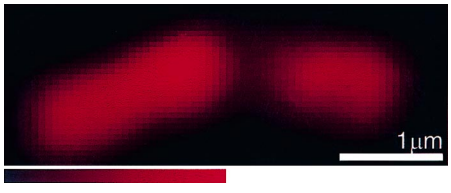

Fig. 4. DsRed expressing *E. coli* imaged with two-photon laser scanning microscope at 1064 nm with an image resolution of 64×30 pixels.

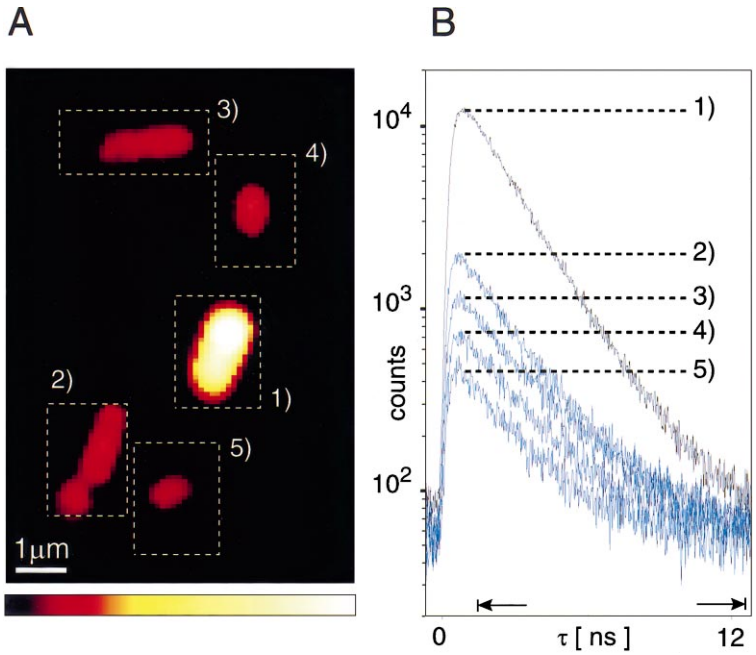

Fig. 5. Fluorescence lifetime microscopy of a fresh mixed *E. coli* culture expressing EGFP or DsRed. A: Fluorescence intensity image. Cell (1) is expressing EGFP, cells (2) to (5) DsRed. B: Corresponding fluorescence decay curves integrated over the designated areas. Arrows indicate the data range used by the fitting algorithm. The resolution and pixel dwell time were 128×64 pixels and 5 ms, respectively.

counterpart, DsRed should be well excitable at about 1000 nm. As EGFP is still excitable at this wavelength [23] we assume that TPLSM with an excitation wavelength of ~1000 nm and optimized filters should be well suited for imaging cells double-labeled with EGFP and DsRed.

3.4. FLIM

As shown in Fig. 5a the FLIM setup can be used to produce fluorescence intensity images of *E. coli* expressing either DsRed or EGFP as with any two-photon microscope. Since we did not discriminate for the different emission wavelengths of the two fluorescent proteins, we used an epifluorescence microscope attached to our setup to clarify that cell (1) is expressing EGFP, whereas cells (2) to (5) DsRed. The corresponding fluorescence decay curves averaged over each of the five cells are shown in Fig. 5b. A difference in the lifetimes of

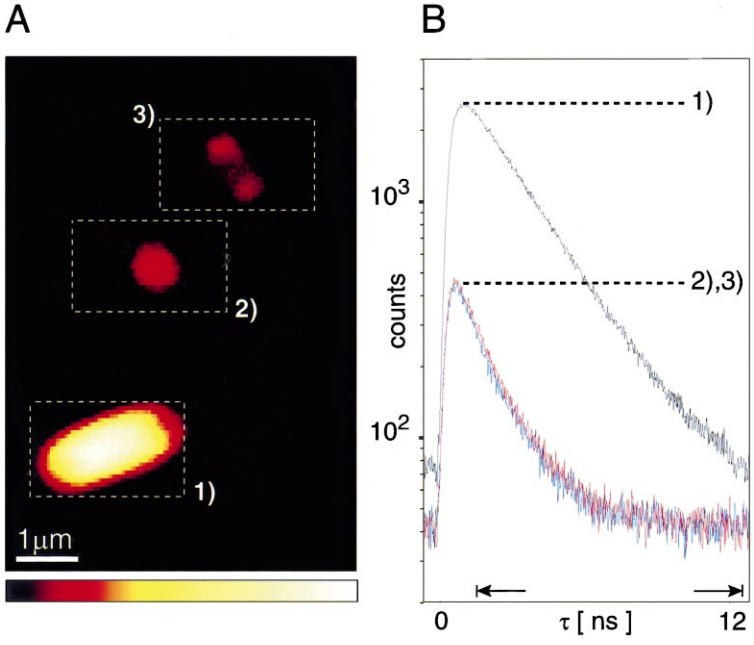

Fig. 6. FLIM of an aged mixed *E. coli* culture. A: Fluorescence intensity image. Cell (1) is expressing EGFP, cells (2) and (3) DsRed. B: Corresponding fluorescence decay curves integrated over the specified areas. Arrows indicate the data sets used by the fitting algorithm. The image has 118×128 pixels and was acquired at 5 ms pixel dwell time.

DsRed and EGFP could easily be recognized from the varying slopes without any further analysis. We fitted the data by a monoexponential decay allowing for a constant background.

In the case of DsRed, the extracted lifetimes varied by up to 150 ps when the fitting range was varied, far more than the statistical error of ~20 ps. This indicates that the single exponential decay only approximates the data and yields an average lifetime. It was however impossible to identify several discrete contributions and the extracted rates most likely constitute the average of a range of values throughout the cell. The decay times extracted from the fit procedure were 2.4 ns for the EGFP expressing cell and 2.7, 3.1, 2.8 and 2.8 ns for the DsRed cells (2) to (5), respectively. Lifetimes of around 2.4 ns for EGFP and between 2.7 and 3.1 ns for DsRed are representative for a larger set of cells ($n > 25$). Our measured lifetime for EGFP of about 2.4 ns agrees very well with the reported ~2.4 ns measured with frequency domain FLIM in live mammalian cells [20]. Fluorescence lifetime measurements could be reliably used to distinguish EGFP and DsRed expressing cells.

We also found that in older bacterial cells DsRed is gradually no longer homogeneously distributed. Importantly, this process is accompanied by a marked change in the lifetime of the fluorophore. The fluorescence intensity image (Fig. 6a) shows an EGFP expressing cell (1) and two DsRed expressing cells (2, 3) of an aged cell culture (held for several days at 4°C). The lifetimes extracted from the corresponding fluorescence decay curves (Fig. 6b) reveal a lifetime of ~2.4 ns for the EGFP and 1.5 ns for the DsRed expressing cells. In a larger set of 'old' cells ($n > 15$) DsRed lifetimes ranged from 1.3 to 1.8 ns. Thus, while the fluorescence lifetime of EGFP was stable for all samples at around 2.4–2.5 ns, the DsRed lifetime decreased markedly from ~2.8 ns to ~1.5 ns over the course of several days.

Further experiments on DsRed cells using excitation at 1064 nm revealed different lifetimes at different locations within the same cell. A detailed explanation for the changes in the DsRed decay time likely requires a comprehensive spectroscopic analysis of DsRed as it has been performed on GFP and its mutants [24,25].

3.5. Conclusion

The fluorescent proteins EGFP and DsRed can readily be expressed in *E. coli*. The pronounced time lag between the appearance of DsRed protein and fluorescence upon induction of protein expression suggests that DsRed has an extended maturation time. We found that DsRed expressing cells are markedly smaller than their EGFP expressing counterparts. DsRed appears to aggregate over time within the cytoplasm, and as such, its putative toxicity needs to be carefully addressed in further studies.

Confocal microscopy with two excitation wavelengths allowed undemanding simultaneous discrimination of cells expressing either of the fluorescent proteins in a mixed cell population. In contrast to previous studies with other GFP-variants no mathematical crossover correction was needed. As any red emitting fluorophore DsRed has the disadvantage that photomultipliers (as well as the human eye) are less sensitive to its longer emission wavelengths, making detection of DsRed fluorescence less efficient. By replacing the PM by an avalanche photodiode this problem might be alleviated [21]. For many applications this disadvantage is probably counterbalanced by its good spectral separability from tissue autofluorescence as well as from other green fluorophores. Although DsRed can be excited at 925 nm, a wavelength of 1064 nm is much more suitable for its two-photon excitation. An excitation wavelength of about 1000 nm should be optimal for dual color two-photon microscopy of EGFP/DsRed double-labeled cells.

An important outcome is that EGFP expressing cells can be distinguished from their DsRed counterpart by their fluorescence lifetime. We found, however, that the lifetime of DsRed decreases with bacterial cell age. This might be due to precipitation of the protein within the cytoplasm, making FLIM measurements of DsRed potentially problematic. Since the DsRed used in this study is the wild type protein, improvements of the protein leading to higher solubility, shortened maturation times, less growth inhibition effects and higher quantum efficiency can be envisaged. These measures will render the combination of DsRed and EGFP a promising option for double labeling and FRET studies in bacterial cells.

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