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Monomeric red fluorescent protein variants used for imaging studies in different species

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Abstract

Fluorescent proteins have proven to be excellent tools for live-cell imaging studies. In addition to green fluorescent protein (GFP) and its variants, recent progress was achieved in the development of monomeric red fluorescent proteins (mRFPs) that show improved properties in respect to maturation and intracellular fluorescence. mRFPmars, a red fluorescent protein designed especially for the use in *Dictyostelium*, has been employed to tag different proteins for live-cell investigations in *Dictyostelium*. mRFPruby, which differs in sequence from mRFPmars in four amino acids, has a codon usage optimised for the application in mammalian cells. Here, we show that both mRFP variants can also be applied for localisation studies in other organisms. mRFPmars was expressed in *Hydra* and fused to the Bcl-2 family protein Bax. mRFPruby in combination with histone 2B was expressed in *Drosophila* S2 cells to monitor mitosis. Using mouse cell lines, mRFPruby fused to β-actin was assayed with high spatial resolution to study details of actin cytoskeleton dynamics. In addition, we demonstrate that both mRFP variants are also suitable for dual-colour microscopy in the different species.

Keywords: *Dictyostelium*; *Drosophila*; *Hydra*; Actin cytoskeleton; Fluorescent protein; Monomeric RFP; B16-F1

Introduction

The advent of fluorescent proteins into cell biology opened a wide field of applications. For more than 10 years green fluorescent protein (GFP) has been used as a tag fused to a variety of different proteins to study their subcellular distribution or dynamics (for review see (Tsien, 1998; Lippincott-Schwartz et al., 2001; Miyawaki et al., 2003; Gerisch and Müller-Taubenberger, 2003)). In recent years, new colour variants of fluorescent proteins have been introduced to expand the spectrum and to allow multi-colour labelling of cells or organisms (Zhang et al., 2002; Shaner et al., 2004; Chudakov et al., 2005). Although a great palette of differently coloured fluorescent proteins is available

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(Shaner et al., 2005), not all are suitable or have been tested in imaging studies. Particularly useful are red fluorescent proteins (RFPs) for multi-labelling studies when sharp separation of emission wavelengths from e.g. GFP is required. Especially the monomeric versions of RFPs provide indispensable tools when dynamic processes have to be imaged. Monomeric RFP1 (mRFP) (Campbell et al., 2002), a derivative of the tetrameric DsRed (Baird et al., 2000), was the first true monomeric RFP and distinguished from previous versions by its improved maturation properties. However, when expressed in cells, for some studies RFP1 proved too dim to acquire images with high spatial and temporal resolution. Since the introduction of mRFP1, several independent approaches in different laboratories led to enhanced monomeric RFP variants.

In one attempt to obtain an improved mRFP for the use in *Dictyostelium* cells, we designed a synthetic gene encoding a variant RFP, designated mRFPmars (Fischer et al., 2004). The mRFPmars gene encoded the mRFP1 amino acid sequence into which six amino acid exchanges were introduced that have previously been shown to improve the brightness of DsRed (Knop et al., 2002). Furthermore, the codon usage of this mutated mRFP gene was optimised for the highly A/T-rich genome of *Dictyostelium*. mRFPmars proved to be a suitable marker for several *Dictyostelium* proteins and also enabled studies with very high spatial and temporal resolution (Diez et al., 2005). However, attempts to express mRFPmars – designed for the use in *Dictyostelium* – in other cell types such as mammalian cells were disappointing. Therefore, an RFP gene encoding mRFPmars with the human codon usage was generated. In an initial screen, a mutated form of mRFPmars, mRFPruby carrying four additional amino acid exchanges, turned out to be even superior to the humanised mRFPmars variant (Fischer et al., 2006).

Here we report on the application of the mRFP variants mRFPmars and mRFPruby in different organisms. We show that the *Dictyostelium* version of mRFPmars can be expressed in the freshwater polyp *Hydra* and is suitable for co-localisation studies. Furthermore, we demonstrate that the humanised variant mRFPruby cannot only be employed in live-cell imaging studies using mammalian cell lines such as mouse melanoma cells, but is also applicable in *Drosophila*.

Materials and methods

Dictyostelium – cell culture conditions and imaging of fluorescent proteins

Cells of the *Dictyostelium discoideum* strain AX2-214 were cultivated at 23 °C in nutrient medium on Petri dishes. AX2-214 cells expressing either GFP-α-tubulin (Neujahr et al., 1998) or GFP-Arp3 (Insall et al., 2001) were transformed by electroporation with a plasmid encoding mRFPmars-LimEΔcoil as described previously (Fischer et al., 2004).

For studying the localisation of GFP and mRFP fusion proteins by live-cell imaging, cells were washed twice in 17 mM K–Na-phosphate buffer, pH 6.0, and transferred to a glass coverslip in an open chamber. Live cells were observed with a confocal microscope (LSM 510 Meta, Zeiss, Germany) equipped with 488-nm argon and 543-nm helium–neon lasers and a 63 × /1.4 NA plan-apochromatic oil objective. For dual-wavelength emissions, BP505-530 and LP585 filters were used in combination with a HFT 488/543/633 dichroic beam splitter.

Hydra – culture conditions, and expression and imaging of mRFPmars

Hydra vulgaris were cultured in *Hydra* medium (0.1 mM KCl, 1 mM NaCl, 0.1 mM $MgSO_4$, 1 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6) at 18 °C. They were fed daily with freshly hatched *Artemia* nauplii. Six to eight hours after feeding, the animals were washed to remove undigested material, which was expelled into the medium (Bosch and David, 1984).

The transfection vector hot Red was constructed by replacing the enhanced GFP (EGFP) coding sequence in the *Hydra* transfection vector hot G (Böttger et al., 2002) with the sequence encoding mRFPmars (Fischer et al., 2004) between the SmaI/EcoRI sites. Fig. 1 shows the resulting vector map for the new vector hot Red. For mitochondrial EGFP expression, the mitochondrial localisation signal from *Hydra* AIF (apoptosis inducing factor; accession number AAX13996.1; GenBank identifier: 60101760) encompassing the sequence

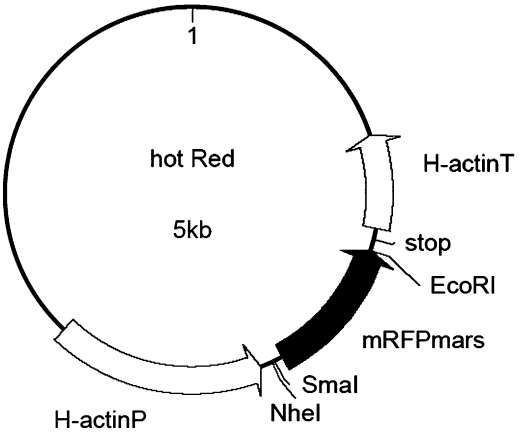

Fig. 1. Vector map for hot Red. The *Hydra* actin promotor (H-actinP) directs strong expression of fusion proteins. The fragment of the sequence can be cloned into the vector either at the N- or at the C-terminus of mRFPmars.

MIRLWKSCSLIRQNGNFICKSIHLKPTTLVRSG-CRYS was fused to the N-terminus of EGFP. For the fusion protein mRFPmars-Bax, the *Hydra* Bax sequence was cloned to the C-terminal end of mRFPmars, using the EcoRI site. Plasmid DNA for transfection was purified using a standard kit (Qiagen, Hilden, Germany) and subsequently precipitated onto 1.0-μm gold particles (Bio-Rad, Hercules, CA). Transfection was carried out using the Helios gene gun system (Bio-Rad) as described previously (Böttger et al., 2002). After transfection, the animals were maintained in culture and examined after 1–2 days for EGFP expression using a stereomicroscope (Leica MZ12, Leica Microsystems, Germany) equipped with a fluorescence module and GFP filter set. For confocal laser scanning microscopy, animals were fixed with 2% paraformaldehyde in PBS for 1 h and mounted on slides with Vectashield mounting medium (Alexis Biochemicals, Burlingame). Optical serial sections were acquired with a confocal laser scanning microscope (TCS SP1, Leica Microsystems) equipped with an oil immersion plan-apochromatic 100 × /1.4 NA objective. The fluorochromes were scanned sequentially. EGFP was excited with a 488-nm argon laser line, and mRFPmars with a 568-nm krypton laser line using an excitation beam splitter TD 488/568/633. Emissions were recorded between 520–540 and 575–625 nm, respectively. The grey-scale single channel images were overlaid to an RGB image assigning a false colour to each channel and then reassembled using Adobe Photoshop 7.0 software (Adobe Systems, USA).

Drosophila – cell culture, expression plasmids and confocal imaging

Drosophila S2 cells were cultured at 25 °C in Schneider's *Drosophila* medium (Gibco/Invitrogen, Breda, The Netherlands) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Greiner Bio-one, Alphen a/d Rijn, The Netherlands), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Breda, The Netherlands).

Vector pAc5.1-H2B-mRFPruby, encoding a fusion of mRFPruby (Fischer et al., 2006) to the C-terminus of histone 2B, was constructed as follows. The coding sequence of the human HIST1H2BJ (H2B) protein (GenBank identifier: 20336753) was amplified from cDNA using the primers 5′-ACAAGATATCGCTATGCCA-GAGCCAGCGAAGTC-3′ and 5′-ACAACTCGAGCT-TAGCGCTGGTGTACTTGGTG-3′. The recognition sites for EcoRV and XhoI are underlined. The coding sequence for mRFPruby was amplified from vector pNCO-mRFPruby using the primers 5′-ACAACTCGAGATGGGCAAGCTTACCATG-3′ and 5′-ACACACGCGTTTAGGATCCAGCGCCTGTGC-TATGTC-3′. The recognition sites for XhoI and MluI are underlined. After restriction with the indicated enzymes, the PCR fragments were ligated in one reaction into EcoRV-MluI-digested pAc5.1/V5-His vector (Invitrogen).

Vector pAc5.1-EGFP-α-Tub85E, coding for a fusion of EGFP to the N-terminus of *Drosophila* α-tubulin, was constructed as follows. The coding sequence of α-Tub85E (GenBank identifier: 24645477) was amplified from total fly cDNA using primers 5′-ACAAGCGGCCGCAC-CATGAGGGAATGCATTTCGGTTCA-3′ and 5′-AC-CCACGCGTTGGCTGTATCATTTGTTGAC-3′. The recognition sites for NotI and MluI are underlined. The PCR fragment was cloned in frame with EGFP in NotI-MluI-digested pAc5.1-EGFP. PCR amplifications were performed using Vent DNA polymerase (New England Biolabs, Ipswich, UK). Oligonucleotides were synthesized by Biolegio (Nijmegen, The Netherlands).

S2 cells were transfected using the $CaCl_2$ method. Briefly, 2.5 μg plasmid DNA were mixed with 10 μl 2.5 M $CaCl_2$, the volume was adjusted to 100 μl with 0.1 × TE (pH 7.6) and this was added drop-wise to 100 μl 2 × HEPES buffer (280 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 50 mM HEPES, pH 7.05) while vortexing. Precipitates were allowed to form for 30 min and then the solution was added to 1×10^6 cells in a 35-mm dish.

For microscopic observation, transfected cells were plated onto coverslips in 35-mm dishes, washed twice with PBS, fixed for 10 min with 3:1 (v/v) methanol/acetone and allowed to dry. Cells were re-hydrated by washing with PBS three times for 10 min. The coverslips were mounted in Mowiol 40–88 (Sigma–Aldrich, Zwijndrecht, The Netherlands) containing DABCO (Sigma–Aldrich). Fluorescence images of histone-2B-mRFPruby were obtained using an inverted confocal laser scanning microscope (TCS SP2, DM RXE, Leica, Rijswijk, The Netherlands) with a 63 × /1.32 NA oil objective. The mRFPruby fusion protein was excited with a 543-nm helium–neon laser line and emission was recorded between 571 and 626 nm using a DD 488/543 dichroic filter.

For time-lapse imaging, cells were cultured and transfected on a round cover glass. The cover glass was transferred to a chamber, especially designed for live-cell imaging on the Leica TCS microscope, and filled with 1.5 ml medium. The chamber was placed into an incubator on the microscope stage, which was kept at 25 °C during the experiment. Images were recorded using the inverted confocal laser scanning microscope described above. Cells were imaged in 5 optical planes every 2 min and followed for a total period of 1 h. EGFP was excited with a 488-nm argon laser line, and emission was measured using a BP 500–550-nm filter. After time lapse analysis, the individual sections were converted to projected images using either the Leica confocal software or Adobe Photoshop 7.0 software.

Mammalian cells – culture conditions, expression constructs, imaging and video microscopy

B16-F1 mouse melanoma cells (ATCC CRL-6323) were grown in DMEM, 4.5 g/l glucose (Invitrogen, Germany) containing 10% foetal calf serum (PAA Laboratories, Austria) and 2 mM glutamine at 37 °C and 7% CO_2. Murine embryonic fibroblast cells immortalized with a temperature-sensitive Simian virus 40 large-T-antigen as described (Lommel et al., 2001) were maintained in DMEM, 1 g/l glucose with 10% FBS (Sigma, Germany) and 2 mM glutamine at 32 °C and 5% CO_2.

For generation of the mRFPruby-actin expression construct, EGFP in an EGFP-C1 vector (Clontech) was first replaced by the human codon usage-optimised variant mRFPruby (Fischer et al., 2006) resulting in pCMV-mRFPruby. Subsequently, the coding sequence of human actin cDNA was ligated into pCMV-mRFPruby to receive pmRFPruby-actin. pEGFP-actin was purchased from Clontech (USA). EGFP-VASP is described in (Carl et al., 1999).

B16-F1 and murine fibroblasts were transiently transfected with SuperFect (Qiagen, Germany) and Metafectene (Biontex, Germany), respectively, according to the manufacturers' instructions. For video microscopy, transfected B16-F1 melanoma and fibroblast cells were replated on acid-washed glass coverslips coated with 25 μg/ml laminin (Sigma) and 50 μg/ml fibronectin (Roche, Germany), respectively.

B16-F1 cells were maintained in an open heating chamber (Warner Instruments, USA) at 37 or 32 °C on an inverted microscope (Axiovert S100TV, Zeiss, Germany) equipped for epifluorescence and phase-contrast microscopy, using 100 × /NA 1.4 plan-apochromatic or 100 × /NA 1.3 plan-neofluar objectives with 1.6 optovar intermediate magnification. The microscope was additionally equipped with electronic shutters (Optilas, Germany) in the transmitted and epifluorescence light paths controlled by a homemade interface and a computer-driven filter wheel (LUDL Electronics Inc., USA) for selective excitation of fluorescent proteins. Tungsten lamps were used for both transmitted and epi-illumination except for the images in Fig. 6C, which were acquired with a mercury lamp (100 W). In single-channel experiments, mRFPruby was routinely observed with a Texas-red filter set (♯41004, Chroma, USA). For dual-colour movies, excitation filters for either EGFP or Texas-red/mRFPruby located in the filter wheel were combined with a dichroic beam splitter and emission filter (XF53, Omega, USA). Data were acquired with a back-illuminated, cooled charge-coupled-device (CCD) camera (TE/CCD-1000 TKB, Princeton Instruments, USA) driven by IPLab software (Scanalytics Inc., USA) and processed using Scion Image 1.62 (Scion Corp., USA) and Adobe Photoshop 7.0 software.

Results and discussion

mRFPmars employed in dual-colour imaging studies in *Dictyostelium*

The RFP mRFPmars has been expressed in combination with several different proteins in *Dictyostelium* cells, and the re-distribution of these fusion proteins was studied by live-cell imaging. In most fusion constructs mRFPmars is hooked onto the N-terminus of the assayed protein, but C-terminal fusions are also feasible (for instance the LimEΔcoil-mRFPmars). mRFPmars-LimEΔcoil and mRFPmars-ABD (actin-binding domain) have already been used to analyse actin cytoskeleton dynamics and reorganization of the actin network during cell motility and chemotaxis (Fischer et al., 2004; Gerisch et al., 2004; Diez et al., 2005). mRFPmars-α-tubulin has been used to follow spindle formation during mitosis (Fischer et al., 2004). Furthermore, mRFPmars-actin was co-expressed together with GFP-actin in *Dictyostelium* cells, and no difference with respect to incorporation into filamentous actin structures was observed, confirming that both fusion proteins can be employed equally well in localisation studies (unpublished). Finally, mRFPmars-histone-2B was used to highlight the chromosomes during different stages of mitosis.

In particular, mRFPmars is useful when used in combination with the "standard" fluorescent protein GFP since the emission spectra of GFP and mRFPmars can be clearly separated. An example for dual-colour imaging of *Dictyostelium* cells is presented in Fig. 2A showing a *Dictyostelium* cell expressing GFP-α-tubulin to label the microtubule network and mRFPmars-LimEΔcoil to visualise filamentous actin. Both structures can be clearly distinguished, and the two labelled proteins have already been employed in this combination to follow cytokinesis in several mutant strains (unpublished). In Fig. 2B, mRFPmars-LimEΔcoil is co-expressed with GFP-Arp3 (Insall et al., 2001), a constituent of the Arp2/3 complex, which is required for the generation of branched actin networks. In regions such as lamellipodia the distribution of the two fluorescently labelled proteins has been studied and exploited to analyse details of cortical actin dynamics employing specific microscopic techniques like total internal reflection fluorescence (TIRF) and spinning disk microscopy (Diez et al., 2005).

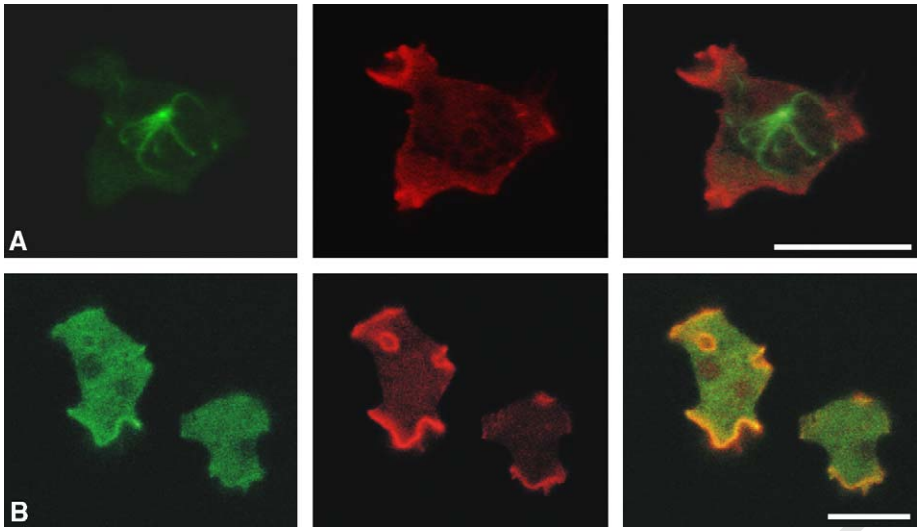

Fig. 2. Dual-colour confocal microscopy of *Dictyostelium* cells expressing mRFPmars-LimEΔcoil (red) to visualise filamentous actin in combination with GFP-labelled proteins: (A) GFP-α-tubulin (green) used to visualise the microtubule network and, (B) GFP-Arp3 (green) to label the Arp2/3 complex. Yellow regions indicate the merge of the two labels. Bars, 10 μm.

mRFPmars is a suitable tool for imaging studies in *Hydra*

Hydra is a member of the phylum Cnidaria and, in evolutionary terms, is one of the oldest multicellular animals. Its simple body plan, its almost unlimited capacity to regenerate and its possession of a pluripotent stem cell line have made it an important model organism for investigating the evolution of animal multicellularity. It is, however, still not easy to conduct functional studies with *Hydra* since it lacks an efficient germ line and due to difficulties in introducing foreign DNA or siRNA into the cells of adult animals. Some of these drawbacks are starting to be overcome, with a recent report detailing single-cell transfections and transient expression of GFP or GFP fusion proteins that enable protein localisation studies (Cikala et al., 2004).

One aspect that is investigated in *Hydra* is the evolution of mechanisms of programmed cell death and their function in maintaining tissue homeostasis in *Hydra*. Members of the Bcl-2 family of pro- and anti-apoptotic proteins are conserved in invertebrates, the evolution of their pro- or anti-apoptotic functions, however, is still not clear (Igaki and Miura, 2004). Many of these proteins are localised to mitochondria via C-terminal hydrophobic tails. However, some pro-apoptotic family members are translocated to mitochondria only during apoptosis (for review see Antonsson, 2001). In order to understand the function of the *Hydra* homologues of Bcl-2 family proteins, it is important to analyse their localisation, their behaviour during apoptosis and their possible influence on mitochondrial morphology. For this purpose it was desirable to introduce a second fluorochrome into *Hydra* cells.

We have now used mRFPmars (Fischer et al., 2004) to express a RFP in combination with EGFP in single cells of adult *Hydra*. The reason to test the *Dictyostelium* mRFPmars variant was the high A/T content of the *Hydra* genome. Both fluorescent proteins, EGFP and mRFPmars, can be co-expressed under the control of the *Hydra* actin promoter. Fig. 3A shows a *Hydra* epithelial cell expressing EGFP and mRFPmars. As observed previously (Böttger et al., 2002), EGFP accumulates both in the cytoplasm and nucleus. Surprisingly, mRFPmars localises only in the cytoplasm and not in the nucleus. This indicates that mRFPmars is not able to passively diffuse into the nucleus in *Hydra* cells, and makes it especially useful for investigating fusion proteins of mRFPmars together with nuclear proteins.

Next, we have used an mRFPmars fusion protein to examine the localisation of the *Hydra* BH3 family protein Bax (for review see Antonsson, 2001). We first constructed a plasmid for expression of a mitochondrial EGFP protein by adding the sequence encoding the mitochondrial localisation signal of *Hydra* AIF to the N-terminus of EGFP. After expression of this construct in *Hydra* epithelial cells, the fusion protein localised to mitochondria, whereas mRFPmars did not (Fig. 3B). Mitochondrial localisation was confirmed by counter-staining of cells expressing mitochondrial EGFP with an antibody against the α-subunit of ATP synthase (Molecular Probes, Oregon) (not shown). Then mRFPmars was fused to the N-terminus of *Hydra* Bax and the resulting fusion protein was co-expressed with

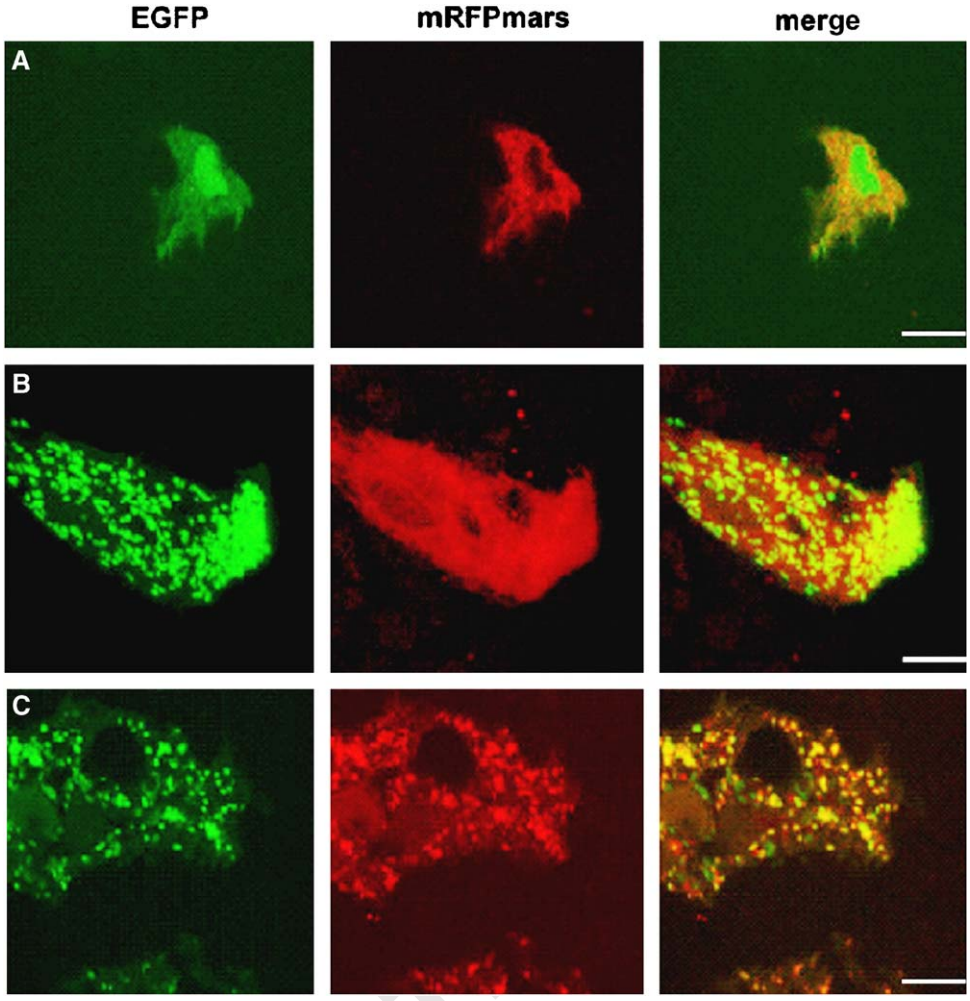

Fig. 3. Expression of mRFPmars and mRFPmars fusion proteins in *Hydra*: (A) *Hydra* epithelial cell expressing EGFP and mRFPmars. Note that there is no co-localisation in the nucleus. (B) *Hydra* epithelial cell expressing EGFP fused to a mitochondrial targeting sequence and mRFPmars. No co-localisation is observed in the merged image. (C) *Hydra* epithelial cell expressing mitochondria-targeted EGFP and mRFPmars-Bax. Both fluorescent fusion proteins localise to mitochondria as indicated by the yellow colour in the merged image. Bars, 10 μm.

mitochondrial EGFP. Dual-colour imaging revealed a complete co-localisation of both ectopically expressed proteins, indicating that *Hydra* Bax associates with mitochondria (Fig. 3C).

These results demonstrate that mRFPmars provides us with a novel tool to study the localisation and redistribution of *Hydra* proteins in single cells and their responses to biological, pharmacological and/or mutational manipulations. Future experiments will include the detailed comparison of mitochondrial morphology in cells overexpressing *Hydra* Bax. Furthermore, the use of mRFPmars together with GFP-tagged fusion proteins will allow co-localisation studies with other members of the *Hydra* Bcl-2 family, in particular *Hydra* Bak and *Hydra* Bcl-2, and will enable the investigation of their dynamics upon apoptotic stimuli.

mRFPruby used for imaging in *Drosophila melanogaster* S2 cells

The fruitfly *Drosophila melanogaster* serves as a well established model organism in a variety of research questions, with an outstanding collection of tools and resources available (reviewed by Matthews et al. 2005). Until now the potential of using mRFPs in *Drosophila* research has only been exploited in one study on asymmetric distribution of fate determinants using histone-2B-mRFP1 (Langevin et al., 2005). This is, however, the only report on expression of any RFP in *Drosophila*. Here the human codon-optimised version mRFPruby (Fischer et al., 2006) was tested for its applicability in *Drosophila* research using Schneider's S2 cells.

To analyse the use of mRFPruby in *Drosophila*, a fusion construct was generated in which the mRFPruby gene was fused to the 3′-end of the human histone 2B gene. Histone 2B is known to bind preferentially to condensed DNA in mitotic cells. Indeed, bright staining of genomic DNA was observed in fixed *Drosophila* S2 cells (Fig. 4). Moreover, all stages of mitosis were observed indicating that the expression of the fusion protein did not interfere with progression through mitosis.

Next we analysed the possibility of using histone-2B-mRFPruby in time-lapse imaging. For proper cell-division, dynamic reorganisation of the microtubule network is required to form the mitotic spindle, which is essential for faithful chromosome segregation between two daughter cells. By expressing *Drosophila* α-tubulin fused to EGFP in combination with histone-2B-mRFPruby, the spatio-dynamic distribution of α-tubulin filaments and segregation of chromosomes was imaged by time-lapse confocal microscopy during mitosis. The average duration of mitosis in S2 cells has previously been reported to be 40 min (De Vries et al., 2005). The progression through mitosis was unaffected in the S2 cells expressing histone-2B-mRFPruby. Fig. 5 shows snapshots in time of a cell going through mitosis from the alignment of the condensed chromosomes at the metaphase plate to a completed cytokinesis (see also Movie 1 in the supplementary online material), demonstrating that the laser intensity required for excitation of the histone-2B-mRFPruby is sufficiently low not to interfere with a sensitive biological process like mitosis.

Expression of mRFPruby in mammalian cells

To test the usability of the human codon usage-optimised variant mRFPruby (Fischer et al., 2006) in mammalian cells, we fused it to human β-actin cDNA, which has previously been exploited as a probe to visualise the actin cytoskeleton in mammalian non-muscle cells (Rietdorf et al., 2001; Steffen et al., 2004). Upon transient expression in the highly motile mouse melanoma cell line B16-F1, mRFPruby-actin was visualised by epi-illumination and found to incorporate into the different subcompartments of the actin cytoskeleton as observed previously using EGFP-actin (Fig. 6A). B16-F1 cells moving on laminin express prominent lamellipodia (arrow in Fig. 6A), microspikes embedded in them (arrowheads in Fig. 6A), variable degrees of stress fibre-like contractile bundles (double-headed arrow in Fig. 6A) and dot- or ruffle-like structures, often appearing in rosette-like arrays (asterisks in Fig. 6A) and known to contain prominent regulators of the actin polymerisation machinery (Stradal et al., 2001). No bias for incorporation into any given actin structure was observed, indicating that ectopically expressed mRFPruby-actin was properly co-polymerised with endogenous actin. No aggregation of mRFPruby-actin in mammalian cells was observed, neither upon expression at levels sufficient for imaging with conventional CCD technology (Fig. 6) nor at higher levels (not shown). The same probe was also studied in cell types other than B16-F1, e.g. fibroblasts,

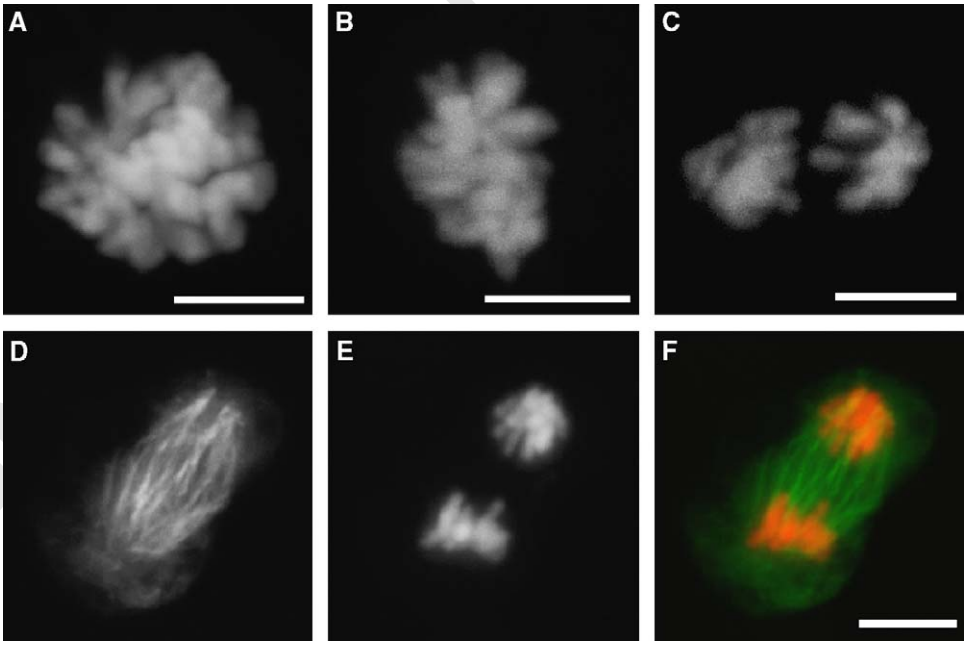

Fig. 4. Expression of mRFPruby in *Drosophila* S2 cells. Confocal microscopy of fixed *Drosophila* S2 cells expressing a histone-2B-mRFPruby fusion protein. The fusion protein was used to visualise the different stages of mitosis: prophase (A), metaphase (B), and anaphase (C). EGFP-α-tubulin (D) was also co-expressed together with histone-2B-mRFPruby (E) in S2 cells. The merged image of (D) and (E) representing a telophase stage is shown in (F). Bars, 5 μm.

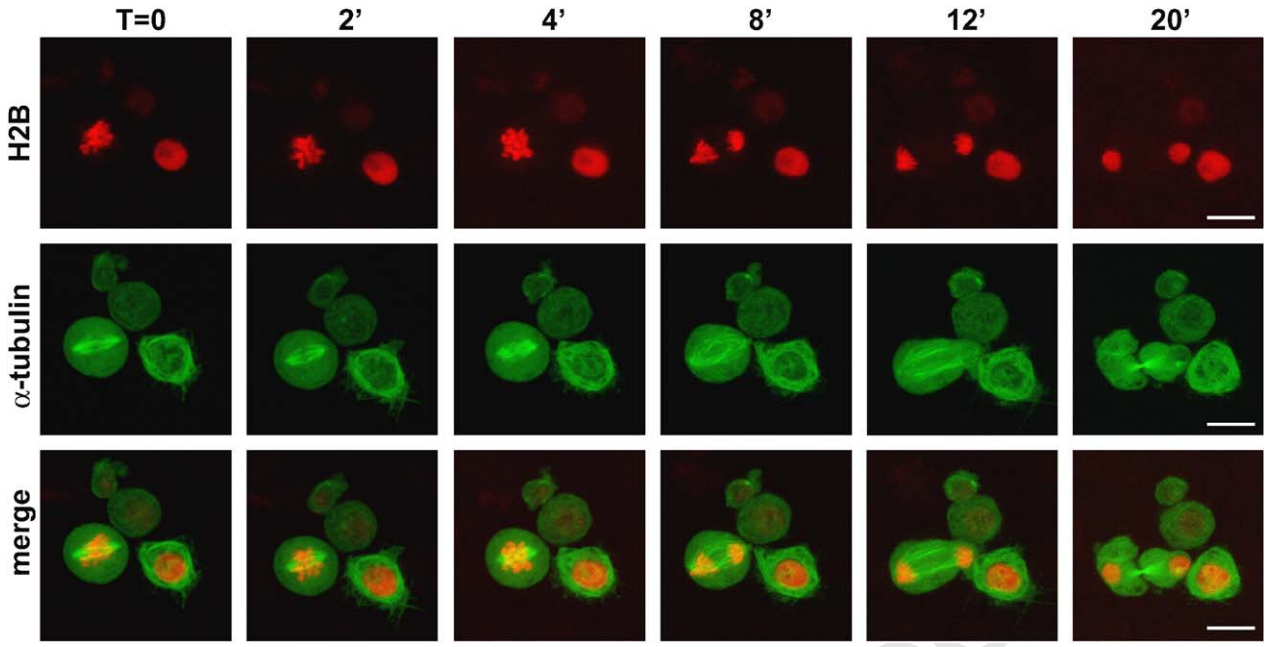

Fig. 5. Time-lapse confocal microscopy using mRFPruby in *Drosophila* cells. The spatio-dynamic distribution of microtubules visualised by GFP-α-tubulin and the segregation of chromosomes visualised with histone-2B-mRFPruby was followed during mitosis of a *Drosophila* S2 cell. Time is indicated in minutes. Stages: 0 min: metaphase, 2–4 min: anaphase, 8–12 min: telophase, 20 min: late mitotic stage. Bars, 10 μm.

with similar results (data not shown), and co-expression of mRFPruby-actin with EGFP-tagged β-actin in these cells revealed incorporation of both probes into the same structures (Fig. 6B).

Finally, we have also examined the usability of mRFPruby for dual-colour live-cell imaging in mammalian cells. The molecular mechanisms of lamellipodia and filopodia protrusion are far from being elucidated (for recent reviews see Small et al., 2002; Pollard and Borisy, 2003; Stradal et al., 2004; Faix and Rottner, 2006). Future efforts to unravel these mechanisms will undoubtedly include a detailed comparison of the dynamics of both constituents and regulators of the actin polymerisation machinery by dual-colour time-lapse microscopy with high temporal and spatial precision. A promising approach to perform such experiments is the employment of probes with excitation and emission peaks fairly distant in the spectrum, such as EGFP and mRFPs (Campbell et al., 2002; Fischer et al., 2004; Shaner et al., 2004, 2005). To test if mRFPruby was suitable for this type of experiments also in mammalian cells, B16-F1 cells were co-transfected with mRFPruby-actin and an EGFP-tagged version of the Ena/VASP family member VASP, which is known to specifically mark active sites of actin polymerisation, such as the tips of lamellipodia and filopodia, focal adhesions or the rosette-like actin arrays mentioned above (Rottner et al., 1999, 2001; Svitkina et al., 2003). Upon transient transfection at a 1:1 ratio, both probes displayed similar brightness and photostability and were well separable with the combination of filter-sets employed (see Materials and methods). As observed previously (Rottner et al., 1999), EGFP-VASP accumulation at the cell periphery was restricted to the very tips of lamellipodia and filopodia/microspikes, but absent from the lamellipodial meshwork (Fig. 6C). Focal accumulation of VASP intensity within the continuous line of VASP localisation at the lamellipodial front corresponded with rapidly translocating microspikes (see also Movie 2 in the supplementary online material), presumably due to increased density of actin filament barbed ends at microspike tips as compared to the rest of the lamellipodium. Microspikes developed by polymerisation from nascent focal actin accumulations at the lamellipodium front, which coincided with high VASP intensities (see Fig. 6C), similar to previous observations (Svitkina et al., 2003). These results further emphasize the feasibility of dual-colour time-lapse microscopy using the EGFP/mRFPruby pair of fluorescent proteins.

Concluding remarks

In summary, this report provides evidence that mRFPmars that originally was designed for the use in *Dictyostelium* can be employed in organisms with a

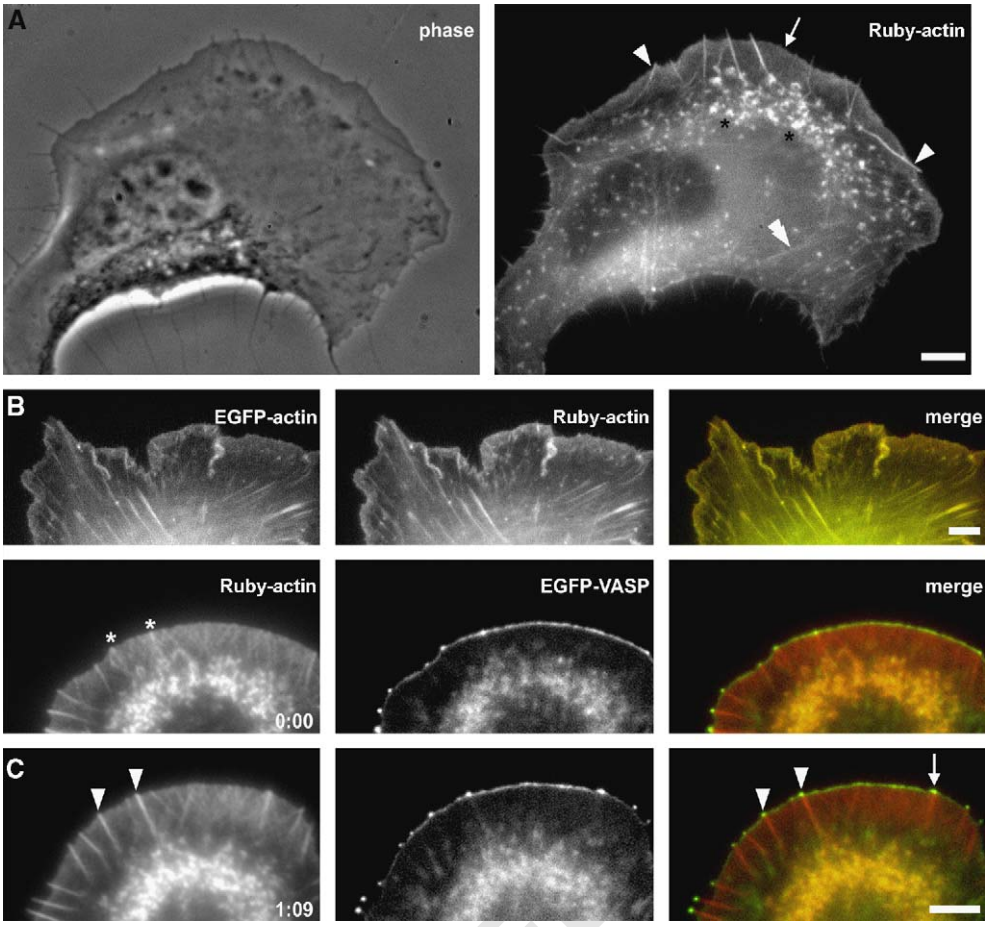

Fig. 6. mRFPruby expression in mammalian cells: (A) B16-F1 transfected with mRFPruby-actin (Ruby-actin) were replated on laminin and observed by phase-contrast and epifluorescence time-lapse microscopy as indicated. Note incorporation of mRFPruby-actin into subcompartments of the actin cytoskeleton typical of motile B16-F1 melanoma cells such as microspikes (arrowheads), lamellipodium (arrow), contractile bundles (double-headed arrowhead) and dots or ruffle-like arrays (asterisks). (B) Identical incorporation of mRFPruby- and EGFP-actin into the actin cytoskeleton of fibroblasts, as emphasized in the merged image (mRFPruby-actin red, EGFP-actin green). (C) Two representative frames of a protruding lamellipodium of a B16-F1 melanoma cell co-expressing mRFPruby-actin and EGFP-VASP as indicated (mRFPruby-actin red and EGFP-VASP green). In addition to the accumulation of VASP at the front of the criss-cross arrangement of lamellipodial actin filaments, the enrichment of the probe also coincided with formation of actin-rich microspike precursors (asterisks in C), which developed into microspike bundles traversing the entire width of the lamellipodium (arrowheads in C). The microspike depicted by the arrow in (C) emerged in between the displayed frames (also see Movie 2 in the supplementary online material). Bars, 5 μm. Times in (C) are in minutes and seconds.

similar codon bias as *Dictyostelium*. Here we presented data on labelling cells of a cnidarian organism, the freshwater polyp *Hydra vulgaris*. This is of particular interest as molecular tools for *Hydra* are at present relatively rare. In this study, mRFPmars was used together with EGFP in mitochondrial co-localisation experiments. It is tempting to speculate that mRFPmars is worth testing also in other organisms bearing a similar codon usage as *Dictyostelium*. For instance, mRFPmars has already been successfully expressed in the protozoan *Paramecium* (Christina Schilde and Thomas Wassmer, personal communication).

Furthermore, the studies presented in this manuscript have also shown that the humanised variant mRFPruby is usable in completely unrelated organisms like *Drosophila* and mouse. In addition to this, mRFPruby has been applied as fluorescent marker in zebrafish for enhancer trapping (Darius Balcunias and Stephen C. Ekker, personal communication). These results encourage further dual-colour imaging studies in higher eukaryotes and flies as the application of mRFPruby allows live-cell recordings without interfering with sensitive biological processes, and thus extends the repertoire of existing RFPs applicable in animal cells.

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The work preceding the results presented in this manuscript was originally started in the lab of Günther

Gerisch a couple of years ago. The primary aim at that time was to create fluorescent probes suitable for high-resolution dual-colour imaging in combination with GFP in *Dictyostelium*. The challenge was to obtain probes stable enough to record images at high video rates and finally led to the development of mRFPmars. The support, constant encouragement and constructive development of ideas by Günther Gerisch is gratefully acknowledged. This work was supported in part by the Deutsche Forschungsgemeinschaft (SPP1150 to K. Rottner and SFB413 to M. Fischer). M.J. Vos is supported by Harm H. Kampinga who received funding from IOP genomics Grant # IGE03018A (Senter Novem, The Netherlands).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2006.05.006

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