Running head: Photo-convertible EosFP probes for plants

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mEosFP based green to red photoconvertible subcellular probes for plants
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Abstract

Photoconvertible fluorescent proteins (FPs) are recent additions to the biologists’ toolbox for understanding the living cell. Like GFP, monomeric EosFP is bright green in colour but is efficiently photo-converted into a red fluorescent form using a mild violet-blue excitation. Here we report mEosFP-based probes that localize to the cytosol, plasma-membrane invaginations, endosomes, pre-vacuolar vesicles, vacuoles, the endoplasmic reticulum, Golgi bodies, mitochondria, peroxisomes and the two major cytoskeletal elements, F-actin and cortical microtubules. The mEosFP fusion proteins are smaller than GFP / RFP based probes and as demonstrated here provide several significant advantages for imaging of living plant cells. These include an ability to differentially colour label a single cell or a group of cells in a developing organ; selectively highlight a region of a cell or a sub-population of organelles and vesicles within a cell for tracking them and understanding spatiotemporal aspects of interactions between similar as well as different organelles. In addition, mEosFP probes introduce a milder alternative to FRAP whereby instead of photo bleaching, photoconversion followed by recovery of green fluorescence can be used for estimating subcellular dynamics. Most importantly the two fluorescent forms of mEosFP furnish bright internal controls during imaging experiments and are fully compatible with CFP, GFP, YFP and RFP fluorochromes for use in simultaneous, multi-colour labeling schemes. Photoconvertible mEosFP-based subcellular probes promise to usher in a much higher degree of precision into live imaging of plant cells than has been possible so far using single coloured FPs.

Key words: EosFP, endomembranes, endosomes, photo-conversion, cytoskeleton, fluorescent proteins, live-imaging.
**Introduction**

Multi-coloured fluorescent proteins (FPs) spanning the entire visible spectrum are considered essential tools for studying gene activity, protein localization and subcellular interactions in modern biology. Numerous subcellular-targeted FP probes have been created for live imaging of plants at the organ, tissue, cell, sub-cellular and sub-organeller levels. Several dedicated web-educational resources have been developed and provide comprehensive and frequently updated information on subcellular-targeted FP probes for plants (Mathur, 2007; Held et al., 2008; Mano et al., 2008; 2009).

The routine use of FPs in plant biology has also made us aware of their limitations. The emission spectra of most commonly used FPs span discrete color bands (Shaner et al., 2007) and consequently all targets of a single FP-fusion become highlighted in a specific colour only. Whereas interactions between dissimilar organelles are readily studied using multicolour labeling using two or more fluorescent proteins (Mathur et al. 2002; Ueda et al., 2004; Kato et al., 2008) single color labeling becomes a limiting factor when the aim is to understand spatiotemporal aspects of interactions between similar organelles. Further limitations of single coloured FPs become apparent when visualizing local and often transient alterations in the organization of dynamic subcellular elements like the cytoskeleton and endomembranes. Carrying out comparisons for these flexible elements simultaneously is challenging and usually not amenable to quantification. Finally an issue that plagues most live-imaging approaches is the absence of built-in controls in the cells under observation. For most researchers the decision of when to stop imaging a cell or a small subcellular region remains empirical rather than one based on a clear imaging parameter. In most studies of living cells internal controls indicating photo-damage are missing as it is generally assumed that such effects must be minimal. Whereas, chlorophyll photobleaching provides a useful visible control in studies involving green tissues a large proportion of published live-imaging data comes from non-green cells and tissues where this internal indicator of cell health cannot be applied. Nevertheless, given the rapid responsiveness of plant cells (Sinclair et al., 2009) internal indicators are extremely important for minimizing artifacts while studying subcellular interactions.

In the majority of transgenic lines created to-date targeted FPs are constitutively

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expressed and cannot be induced at will. However, as underscored through studies utilizing heat shock and chemically inducible promoters (Saidi et al., 2005; Ketelaar et al., 2004; Tang et al., 2004) FP-inducibility is a very desirable trait for studying organelle-interactions and protein-protein interactions. It is noteworthy that for most inducible systems developed so far the induction is global, often involves multiple tissues or entire seedlings and cannot be easily switched off. Frequently, expression levels fluctuate over time and can lead to cumulative protein over-expression related artifacts.

In its present stage the field of imaging subcellular events and interactions in living plants could greatly benefit from monomeric FP-probes that combine the favorable properties of existing FPs with rapid, irreversible photo-convertibility. More important, photo-convertible probes should work under existing microscopy infrastructure without requiring additional monetary inputs, be compatible with existing FP-probes and provide quantifiable data. An invaluable quality sought in the new probes should allow their use as internal controls for monitoring cell health during live-visualization.

Recently several new ‘optical highlighters’, broadly categorized as photo-activatable, photo-switchable and photo-convertible FPs have become available (Ai et al., 2006; Shaner et al., 2007; Wiedenmann et al., 2009). In response to specific wavelengths these proteins undergo structural changes that result in their becoming ‘switched on’ to a bright fluorescent state (photoactivatable FPs; Patterson and Lippincott-Schwartz, 2002) or cause a shift in their fluorescence emission wavelength (photo-convertible FPs; Wiedenmann et al. 2004; Gurskaya 2006). The use of photoactivatable GFP (PA-GFP), photo-convertible Dendra and Kaede has been successfully demonstrated for plants (Arimura et al., 2004; Runions et al., 2006; Martin et al., 2009; Brown et al., 2010). Moreover, EosFP, a homolog of Kaede derived from Lobophyllia hemprichii has been engineered to a monomeric form without loss in fluorescence and photoconversion properties (Wiedenmann et al., 2004; Nienhaus et al., 2005) and utilized for demonstrating clathrin-dependent endocytosis during internalization of PIN auxin efflux carriers (Dhonukshe et al., 2007), for labeling F-actin (Schenkel et al., 2008) and peroxisomes (Sinclair et al., 2009) in plants. In its unconverted form mEosFP displays bright green fluorescence that upon illumination with ca. 390-405 nm waveband changes irreversibly
Here we present several new mEosFP based probes targeted to the general cytosol, plasmalemmasomes, endosomes, tonoplast, endoplasmic reticulum, Golgi bodies, peroxisomes and cytoskeletal elements. The collection includes LIFEACT::mEosFP, a small, most recently developed F-actin marker. The parameters used by us for assessing mEosFP probes include their expression in different cell types, under transient and stable conditions and extend to developing optimal and economically feasible conditions for visualizing these probes. We demonstrate their usefulness in tracking single organelles over time, in following membrane and cytoskeletal dynamics and their compatibility and ease of visualization with other coloured FPs while pointing out potential pitfalls and artifacts that might result during visualization. In addition the discovery of tubular endosomes resulting from mEosFP-based differential coloration of specific vesicles is described. Our detailed characterization of mEosFP-based probes suggests their tremendous potential in advancing spatiotemporal precision during live imaging of plants.

**Results and discussion**

**mEosFP based probes: Ease and specificity of photo-convertibility**

Monomeric EosFP (mEosFP; Wiedenmann et al., 2004; Nienhaus et al., 2005) was found to be bright fluorescent green in both transient assays and in transgenic plants (Fig. 1). The protein photo-converted easily to a vivid red color following a 3-7 second exposure to violet-blue illumination (Fig. 1). Different microscopy setups (see materials and methods) could be used for photo-conversion. These included a conventional DAPI filter (Ex. 350/50X; CdLP 400; Em. 460/50 nm; setup 1 and 4; materials and methods), a violet-blue glass filter (Leica D-filter; Ex. BP 355-425/dichroic 455/ LP 470 nm; setup 2; materials and methods) on epi-fluorescent microscopes and a 50 mW variable power 405 nm laser (setup 3; materials and methods). The automated FRAP module on a Leica CLSM TCS-SP5 was tested and found to provide adequate photo-conversion within 3-5 seconds using a 70% laser strength. However, practical considerations such as cost constraints associated with purchasing a dedicated 405 nm laser made us assess existing epi-fluorescent microscopes where photo-conversion required manual opening and
shutting of the diaphragm. For each probe (Figures 1-4) a conventional DAPI filter or a violet-blue D-filter allowed us to achieve the same degree of photo-conversion as the high-end 405 nm laser equipped CLSM. Since epi-fluorescent setups usually do not provide beam diameters smaller than 500 µm we found a simple solution by custom creating smaller pinholes of 100 and 50 µm on our Leica DMRE microscope epi-fluorescent microscope.

Thus where available, the 405 nm laser should be considered the preferred mode for photo-conversion as it allows a high degree of precision through associated automated controls. However, lack of the 405 nm laser does not limit use of these probes as conventional filters on epi-fluorescent microscopes can be used for photo-conversion. To allow complete reproducibility all the photo-conversions shown in images presented here have been carried out using glass filters on epi-fluorescent microscopes.

Properties, uses and caveats associated with various mEosFP probes in plants:

A number of mEosFP fusion probes targeted to different subcellular compartments and structures are reported (Table 1). While demonstrating photo-convertibility for each probe we have utilized some of them for establishing the novel properties, associated caveats and potential uses of mEosFP in plant research.

Transgenic Arabidopsis plants expressing the mEosFP-cytosolic probe (Fig.1) were used for assaying the stability of both coloured forms of mEosFP. Observations on more than 70 different transgenic lines harboring mEosFP-cytosolic did not reveal growth and reproductive abnormalities suggesting that the protein is well tolerated by plants. Photo-conversion of single epidermal cells provided clear differentiation between the green and red forms of mEosFP (Fig 1 A-F). Differences were also seen between the red form of the protein (R-mEosFP; Fig. 1 D) and chlorophyll auto-fluorescence (false coloured blue; Fig. 1D) by collecting emission spectra between 570 and 620 nm and 626 to 763 nm, respectively. Both sequential as well as simultaneous use of 488 nm and 543 nm lasers during confocal visualization allowed constant monitoring of the non-photoconverted green fluorescent form (G-mEosFP) of mEosFP within a cell. This provided the desirable internal control during live-cell imaging since photo-bleaching of
G-mEosFP from non-photoconverted regions was an indication of compromised cellular health. In all subsequent visualization reported here using different mEosFP-based probes this internal control was rigorously maintained. It is noteworthy that in cells that have high levels of mEosFP expression a short photo-conversion period will allow colour change to be readily visible but will invariably leave a certain residual green fluorescent form of mEosFP also (Fig.1A versus C). Care must be taken to record this residual form in the first scan after photoconversion. This is important as in subsequent scans with a 488 nm laser, depending upon the laser strength this partially activated form can either become photo-bleached or fully photo-converted. A fluorescence intensity line tracing of RGB values within the photo-converted region (inset-Fig. 1A-D) usually helps to resolve whether the change involves photoconversion or photo-bleaching. In contrast to the situation in Fig. 1 D, during photo-bleaching the decrease in green fluorescence is not accompanied by a concomitant increase in red fluorescence. If a slight increase is observed in chlorophyllous cells it is usually attributable to photo-bleached chloroplasts. In general, a minimal 488 nm laser power (1-5 %) and a high (ca. 60-80%) 543 nm laser power allowed us to avoid extra photoconversion as well as photo-bleaching while providing a high green to red contrast. In symplastically isolated epidermal cells such as mature trichomes (Fig. 1E) and guard cells (Fig. 1F) the R-mEosFP is very stable and can be easily detected even after 48 hours.

While observing photo-converted cells in mEosFP-cytosolic plants a general artifact that relates equally to all photo-convertible probes became apparent. The diameter of an excitation beam notwithstanding, the illumination and consequent photoconversion of G-EosFP occurs in a concentric manner where the intensity of the excitation wavelength disperses around the beam focal point. Thus under short exposure time G-mEosFP molecules at the periphery might be photo-converted only partially in comparison to the those lying on the precise focal point. This slight variation in distribution of excitation intensity results in mEosFP hues ranging from yellow to orange-red at the periphery (Fig.1F). Therefore it is important to recognize the minor variability in shades of red that occurs during green to red photoconversion and use the maximal red as a point of reference. Fig. 1F where the photo-conversion was carried out on the top guard cell using
a circular beam (broken white lines) demonstrates this color variability. A green fluorescent nucleus unexposed to the maximal intensity of photo-converting light did photo-convert completely and therefore appears yellow rather than red or green (Fig. 1F). A histogram of RGB values (inset -Fig. 1 F) across the red to green region (ROI) underscores this point while color bar on the side clearly depicts the mixing of red and green colors to produce a variety of hues. Awareness of this artifact is especially important when using mEosFP in studies involving protein co-localization since a partial photo-conversion as well as full co-localization will both result in similar yellow hues. However, when 488 nm and 543 nm laser lines are used together the green moiety in partially photo-converted cells usually photo-bleaches faster (within a few scans) and the yellow colour changes to a stable red. Therefore protein co-localization should be inferred only if a yellow signal persists after multiple laser scans.

Another artifact relating to the use of monomeric EosFP became apparent in mEosFP-cytosolic plants grown in bright fluorescent white light (ca. 80-100 μmol m⁻² sec⁻¹) where up to 25% hypocotyl epidermis cells contained red nuclei (Fig. 1G) even without intentional photo-conversion. Notably, monomeric EosFP (mEosFP -226 amino acids; ca. 25.8 kDa) is slightly smaller than monomeric GFP (238 aa; ca. 26.9 kDa). Like non-targeted GFP (Haseloff et al., 1997) mEosFP highlights the entire cytosol and apparently diffuses freely in and out of the nucleus (Fig 1A, B). The artifact might thus be attributed to a combination of high violet-blue component in the fluorescent-white light spectrum and a possible higher concentration of mEosFP within the nucleus of certain endoreduplicating hypocotyl epidermal cells (Gendreau et al., 1998). Similarly, cells transiently expressing mEosFP-cytosolic and injured cells on transgenic plants were often found to contain bright fluorescent nuclear aggregates. Thus care must be taken to check cells under both GFP/FITC and RFP/TRITC filters before photoconversion. Additional care is required in interpreting the non-targeted nuclear localization of mEosFP-cytosolic as after photo-conversion its bright red nuclear fluorescence often over-shadows the fainter cytoplasmic fluorescence.

All components in a living plant cell show dynamic behaviour. While observations on organelles such as mitochondria, plastids, peroxisomes and Golgi bodies
are aided to a great extent by their well-defined morphology the shape of endomembrane compartments is not fixed. In striving to adjust the subcellular milieu to the external environment, endomembranes are in constant flux. Endomembrane compartments frequently form transient contacts, glide over each other and frequently form tubules and vesicles of assorted shapes (Harris 1986). Selective labeling and tracking of endomembrane compartments presents a major challenge for live imaging. A number of probes described here (Table 1) have therefore been targeted to endomembrane compartments for assessing the utility of mEosFP for tracking vesicles and estimating changes in membrane dynamics.

**mEosFP probes allow highly specific differential labeling of membranes and vesicles**

The plasma membrane is the outer limiting membrane of the plant cell but maintains high connectivity with the cell interior through tubulo-vesicular compartments known as plasmalemmasomes or plasma-tubules (Robinson et al., 1996). The aquaporin PIP1 (plasma membrane intrinsic protein 1; Fetter et al., 2004) has been shown to be enriched on paramural plasma membrane invaginations (Robinson et al., 1996) and was used for generating the probe for plasmalemmasomes / plasmataubules. Transient expression of the mEosFP::PIP1 probe in onion epidermal cells highlighted a convoluted plasma-membrane as well as internal tubular-vesicular compartments, including numerous multi-lamellar vesicles of different sizes (Fig. 2A). Morphologically, the highlighted compartments closely match the electron micrographs of plasmalemmasomes (Harris et al., 1982; Robinson et al., 1996). The presence of multi-lamellar vesicles is also consistent with the observations of Marchand and Robards, (1968) who had considered plasma-membrane evaginations as precursors for multi-vesicular bodies possibly associated with cell-wall formation. Individual mEosFP::PIP1 vesicles were photo-converted and maintained their colour-differentiated status over 12 hours of visualization.

From the cell-interior perspective vacuoles are able to maintain an optimal water homeostasis within the cell through constant communication with the cell boundary. Aquaporins belonging to the TIP1 (tonoplast intrinsic protein 1) subfamily of major
intrinsic proteins (MIPs) have been shown to localize to the vacuolar membrane (Cutler et al., 1999; Ma et al. 2004; Hunter et al., 2007). The localization of mEosFP::α–TIP1 matched observations of Hunter et al. (2007) as it labeled the tonoplast of large central vacuoles in onion epidermal cells (Fig. 2 C, D). However, in our transient expression assays endomembranes highlighted by mEosFP::α–TIP1 included smaller vacuoles (Fig. 2 C arrowhead) and tubular strands. Photo-activation of small domains on vacuoles followed by time-lapse observations showed the flexible nature of the vacuolar compartment but also raised the possibility that α–TIP1 localization might be similar to that reported for AtTIP1;1 a gamma TIP that localizes to the tonoplast junctions (Beebo et al., 2009). Transgenic plants required for further investigating this possibility are being created.

Different early and late endosomes/multi-vesicular bodies and pre-vacuolar vesicles traffic between the cell boundary and large vacuoles (Bethke and Jones, 2000; Lam et al., 2007; Robinson et al., 2008). A third probe with high potential for investigating relationships between different endo-membrane compartments was created by fusing mEosFP to two tandem FYVE domains. The FYVE domain, specifically binds the phosphoinositide PI(3)P (PtdIns3P), a key player in membrane trafficking in animals, yeasts and plants (Burd and Emr, 1998; Gaullier et al., 1998; Gillooly et al., 2000). As observed with other FP::2xFYVE fusions (Voigt et al., Vermeer et al., 2006 ) the mEosFP::2xFYVE probe highlighted numerous vesicles ranging in size from 200 nm to 25 µm in diameter (Fig. 2E). Based on prior publications (Kim et al., 2001; Voigt et al., 2005; Lam et al., 2007) and our unpublished co-localization (data not shown) with the commonly used endosome labeling steryl dye FM4-64 (Vida and Emr, 1995) the smaller vesicles (box Fig. 2E) were considered to be endosomes. As suggested by Vermeer et al., (2006) the larger vesicles were interpreted as pre-vacuoles or vacuoles (Fig. 2E). Since small vesicles move rapidly within the cell a 3 second exposure resulted in varying degrees of photo-conversion. Fig. 2E illustrates the multi-colour labeling that was achieved in this manner. The observation suggests a correlation between FP content and the color of a vesicle. Exposure of pre-vacuoles containing one or more internalized vesicles lent further support to this conjecture. A single exposure produced multicolored vesicles inside a larger vesicle
These observations suggest that short pulses of photo-converting wavelength can be used to estimate the relative concentrations of proteins in vesicles in living cells. Alternatively as single vesicles of more than 2µm could be easily photo-converted separately (Fig. 2G) we wondered whether the mEosFP::2xFYVE probe could be used for assessing homotypic vesicle fusion. Vesicle fusion takes place under normal conditions and is responsible for the growth and development of large vacuoles. The process is greatly augmented during stress. mEosFP::2xFYVE labeled vesicles were photo-converted within a small region of the cell and allowed to mix for 5 minutes before the cell was challenged with 500 mM salt (NaCl). As shown (arrowhead Fig. 2G) membrane fusion occurred along lines of contact during the salt induced plasmolysis. Interestingly a treatment involving 10% poly-ethylene glycol-6000 resulted in aggregation of small vesicles around larger ones (Fig. 2H) with rapid fusion taking place upon de-plasmolysis following PEG removal. The observations suggest that both forms of mEosFP are stable under low hydration conditions and mEosFP::2xFYVE can be used as an experimental tool for understanding membrane fusion.

Although different XFP::2xFYVE probes have been reported to label endosomes and vacuoles (Kim et al., 2001; Voigt et al. 2005; Vermeer et al. 2006) there are no reports of their highlighting long membrane tubules. In our observations on onion cells transiently expressing the mEosFP::2xFYVE probe we invariably observed tubular connectivity between large vesicles. Fig. 3A shows tubules of varying diameters (ranging from 0.25 to 1.5 µm) extending between large pre-vacuolar vesicles. Since their initial observation in onion epidermal cells we have been able to confirm similar tubules extending over the vacuolar surface in transgenic Arabidopsis plants expressing GFP/RFP/mEosFP::2xFYVE probes. Photoconversion of single vesicles allowed an associated tubule to achieve the same colour within 10 – 15 seconds. However tubules that appeared to be continuous maintained distinct colors (facing arrowheads Fig. 3A) suggesting that their ends are sealed and do not create a continuous compartment.

mEosFP aided vesicle tracking reveals a unique FYVE -labeled compartment
The ability to differentially highlight a few vesicles amongst hundreds of similar vesicles led to a serendipitous observation. Sporadically a few mEosFP::2xFYVE labeled vesicles displayed an aberrant oscillatory-rotatory motion. These aberrantly behaving vesicles were photo-converted and found to extend tubular projections. The asymmetrically shaped vesicles rapidly elongated into narrow tubules with lengths ranging between 5 to 15 µm and a diameter of 0.6 ± 0.13 µm (Fig. 3B). The photo-converted tubules could be easily tracked between non-photo-converted mEosFP::2xFYVE labeled vesicles. Tubules frequently formed loops and appeared to snare other vesicles into lose aggregates (Fig. 3C, D). Ensnared vesicles finally fused with the tubules. The persistence of tubules within a cell varied considerably, ranging from a few minutes to up to 4 hours. New tubules were constantly generated while all tubules invariably re-circularized to form spherical vesicles again. Although our observations do not provide an immediate explanation for these tubular compartments and their snaring of vesicles it is noteworthy that in animal cells early and recycling endosomes are often described as tubulo-vesicular compartments that accumulate internalized cargo and display tubular extensions (Tooze and Hollinshead, 1991; Gruenberg, 2001; Miaczynska and Zerial, 2002). Similar membrane extensions from either endosomes or pre-vacuolar compartments have not been reported in plant cells so far (Otegui and Spitzer, 2008). The transient and sporadic occurrence of these membrane extensions may explain why they have not been reported in earlier studies involving FP fusions with different SNAREs, Rab GTPases and plasma-membrane proteins that have formed the basis of our views on the endosomal compartments in plants (Sönnickshen et al., 2000; Geldner et al., 2009). As an alternative explanation, some of the tubular structures appear to resemble the cup or bulb shaped structures observed by Beebo et al., (2009). However, the majority of tubules reported here do not appear to fall into similar categories. The oscillatory movement that initially drew attention to a few vesicles and made us photo-convert them probably resulted from a growing asymmetry in vesicle shape due to localized tubular projections. Despite the asymmetry-driven aberrant motility these vesicles continued to provide a generally circular profile that would have been easily missed amongst the hundreds of similarly fluorescent vesicles in a cell. Photoconversion also confirmed that the vesicles carried the mEosFP::2xFYVE label and
are not imaging artifacts. Since their initial visualizations in onion cells we have been able to observe similar short lived tubules in transgenic Arabidopsis plants expressing XFP::2xFYVE and confirm that these are not artifacts of transient expression. While an element of serendipity is apparent in our observations the discovery of tubular endosomal compartments in plant cells can directly be attributed to our ability to differentially highlight a subpopulation of green fluorescent vesicles and follow them over time. Photoconvertible mEosFP clearly has tremendous potential for unveiling new and novel subcellular compartments and activities through differential highlighting. Follow up studies on these transient tubular compartments are being pursued in stable transgenic Arabidopsis plants expressing mEosFP::2xFYVE and will be reported separately (Mathur et al. unpublished).

ER targeted mEosFP provides a mild method for assessing endomembrane dynamics

The cortical ER is composed of labile extending and retracting, anastomozing membrane tubules, fenestrated sheets as well as compact spindle shaped ER bodies (Matsushima et al., 2003; Hara-Nishimura et al., 2004). The dynamic nature of the ER suggested a way of using mEosFP for assessing rapid membrane flow and reorganization. The mGFP-ER construct (Haseloff et al., 1997) has been one of the most used probes for plants. Using similar constructs the ER has also been successfully visualized in other colors (Sinclair et al., 2009). However, ss::mEosFP-HDEL is retained within the ER lumen and does not allow the ER membrane to be visualized specifically. The PAGFP-ER construct consisting of trans-membrane domains of Arabidopsis calnexin (Huang et al., 1993) fused to a photoactivatable GFP has been reported by Runions et al., (2006) to efficiently label the ER membrane. It formed the basis for creating the CX::mEosFP probe. Unlike PAGFP-ER, which is nearly non-fluorescent prior to its photo-activation the non-photo-converted CX::mEosFP is bright, fluorescent green (Fig. 4A-top). It thus provides a significant advantage over the parental probe. CX::mEosFP photo-converts readily (Fig. 4A-bottom) and efficiently highlights rapidly moving membrane vesicles, fenestrated membrane sheets and cortical ER tubules while negatively highlighting ER bodies, and the nucleus. In general, the green form of mEosFP is irreversibly photo-converted to the red form.
(Weidenmann et al., 2004 and Fig. 1E) and thus color dilution can be attributed to protein dispersal, membrane flow and reorganization. In healthy plant cells the rapid movement of CX::mEosFP creates a quick merging of green and red fluorescence. As shown in Fig. 4B the localized photo-conversion of CX::mEosFP creates a color range between green and red. CX::mEosFP was used to estimate membrane flow from one subcellular locale to another by analyzing the ratio of un-converted, green CX::mEosFP to its red form (Fig. 4C, D). Recovery of green fluorescence in the same region can be used to estimate membrane and fusion protein dynamics (Fig. 4 D). The applicability of the method for providing correlative measurements is further demonstrated using BDM and Latrunculin-B aided inhibition of ER motility (Fig. 4 E). Acquisition of similar data can be useful for interpreting the mobility and behavior of other organelles. The method using mEosFP-probes and assessing colour recovery after photo-activation follows the same principle as the commonly used FRAP (Fluorescence Recovery After Photo-bleaching; Reits and Neefjes, 2001) method for measuring protein mobility and activity in living cells. However, it promises a significant advantage over FRAP since the harsh photo-bleaching step is replaced by the much gentler photoconversion of mEosFP. A major point to be considered in the color-based approach relates to color conversion efficiency (CCE). This may be defined as the ratio of green and red forms of mEosFP obtained following a colorimetric RGB scale of 0 to 255 values plotted over time. The CCE depends upon the initial amount of fusion protein in a cell as well as its turnover over a specific period. Thus the photoconversion of protein accumulated within a small compartment might be quite rapid but depending upon the rate of turnover its subsequent recovery might take a long time. For certain proteins the cytoplasmic diffusion rates might be quite considerable and would need to be taken into account. CCE will also vary with the photoconversion filters / laser and the focal intensity of the excitation beam. Nevertheless as long as observations are made under the same conditions the method should allow direct comparisons of digitized images.

Small mEosFP based cytoskeletal probes:
Both actin filaments and microtubules create very flexible and highly responsive arrangements on the cell cortex. In recent years several probes have been created for labeling F-actin and each new probe has provided a better appreciation of the dynamic nature of the actin cytoskeleton. We have reported a mEosFP::FABD-mTalin probe previously and demonstrated the successful labeling and photoconversion of F-actin in living plant cells (Schenkel et al., 2008). Nevertheless, smaller probes that result in minimal interference with actin dynamics are still sought. A 17 amino acid peptide called LIFEACT (amino acid sequence MGVADLIKKFESISKEE) from the yeast Abp140p fused to a GFP or RFP has emerged as the smallest live-probe for labeling the actin cytoskeleton (Reidel et al., 2008). LIFEACT::XFP has already been tested in several systems, including plants and reportedly does not interfere with actin polymerization or depolymerization. Reportedly its use does not influence the major actin dependent processes of polar tip growth in lower and higher plants (Era et al., 2009; Vidali et al., 2009). The LIFEACT::mEosFP fusion presented here (Fig. 5A, B) is only 246 aa (ca. 28 kDa) as compared to LIFEACT::GFP (263 aa / ca. 30 kDa; Reidl et al., 2008); GFP-mTalin (447 aa / ca. 49 kDa; Kost et al., 1998); ABD2::GFP (70 kDa; Sheahan et al., 2004; Hofmann et al., 2009). It is much smaller than the other photoconvertible F-actin highlighting probe mEosFP::FABDmTalin (425 aa / ca. 47 kDa; Schenkel et al., 2008) and thus combines two highly desirable properties. The potential of LIFEACT-mEosFP for estimating localized alterations in F-actin organization are evident from Fig 5E. Whereas the change in morphology of F-actin strands might reflect their rearrangement during cytoplasmic streaming the ability to quantify changes within an actin strand is a powerful method to understand local actin dynamics (Watanabe and Mitchison, 2002; Ponti et al., 2003). As shown in Fig. 5 F changes in the green:red ratio within an actin cable can be observed and quantified. Progressive quantification of a green or red region suggests a viable method for in vivo speckle labeling and determining actin filament polarity (Fig. 5 F). Although the LIFEACT marker can readily release from actin filaments and rebind at other positions (Reidel et al., 2008), it is suggested that with refined controls LIFEACT-mEosFP can become a useful addition to existing fluorescent speckle microscopy methodology (Danuser and Waterman-Storer 2006). However, these are early stages of
LIFEACT use in plants and there could be potential pitfalls that will become apparent only upon wider usage.

The fusion of different FPs to the microtubule-binding domain of the mammalian MAP4 gene has been very useful for observing cortical microtubules and their dynamics in plants (Marc et al., 1998; Mathur et al., 2000). Due to the smaller size of mEos the mEosFP::MBD-MAP4 version (Fig. 5C,D) is slightly smaller than similar CFP/GFP/YFP/RFP/ probes and has the added feature of photo-convertibility. Whereas a number of transgenic lines expressing GFP-MDB-MAP4 exhibit microtubule bundling (Marcus et al., 2001) the transgenic Arabidopsis plants expressing mEosFP::MBD-MAP4 do not exhibit this defect so far.

Using mEosFP to understand organelle behaviour and interactions

The probes mito-mEosFP (Fig. 6 A, a, B, b) and mEos::GONST1 (Fig. 6 C, c) target to mitochondria and Golgi bodies, respectively. While probes with similar targets have been created using KaedeFP, a tetrameric homolog of EosFP (Arimura et al., 2004; Brown et al., 2010) the two probes developed by us use the monomeric form.

However, a potential artifact must be pointed out when using photoconvertible FPs to understand organelle interactions. Amongst other parameters such studies rely heavily on demonstrating co-localization between two organelles (eg. Arimura et al., 2004; Fig.6 A, a, B, b). Notably, in living cells, organelles like mitochondria and peroxisomes are highly motile and thus even a minimal lapse in the timing of the capture of sequential red or green images (caused by single-band cube switching when using broad-spectrum illumination) can lead to the appearance of a lack of co-localization, as a result of a given organelle moving in the x, y, or z planes between exposures. As depicted in the inset in Fig. 6B merged images may produce a green signal that could be interpreted as a lack of co-localization, when in fact there may be complete co-localization. Laser scanning acquisition allows simultaneous fluorescence excitation but, depending on the method of image capture, a single fast-moving organelle may appear multiple times in the same image. Unambiguous imaging of multi-coloured fast-moving organelles therefore necessitates either the use of a colour camera, or the use of a beam splitter.
The GONST1 gene of Arabidopsis encodes a Golgi localized GDP-Mannose transporter (Baldwin et al. 2001) and its full length cDNA was used to create mEosFP::GONST1. As shown for the GONST1::YFP fusion (Baldwin et al. 2001) the mEosFP::GONST1 probe labeled motile punctate bodies (Fig. 6C), that photo-converted readily (Fig. 6c). Recently Golgi-targeted fusions with a tetrameric Kaede protein have been reported (Brown et al. 2010). Over-expression of oligomeric proteins usually results in aggregates ranging in size from 0.5 to 2.5 µm and thus limits their suitability for visualizing small organelles, which fall within the same size range (Weidenmann et al., 2004). Whereas intuitively the monomeric form of a protein should be considered superior to its tetrameric one for live imaging purposes the relative pros and cons of using mEosFP probes or Kaede based probes require further investigation.

mEosFP is fully compatible with other coloured FPs

For mEosFP-based probes to be truly useful in multi-colour live imaging they should be compatible with FP of other colors such as CFP, GFP, YFP, and RFP. This was tested using mEosFP probes targeted to Golgi bodies and the previously described mEosFP-PTS1 probe targeted to peroxisomes (Sinclair et al. 2009). The simultaneous visualization of mEosFP::GONST1 with GFP-mTalin (which labels F-actin; Fig. 6C, c), mEosFP-PTS1 with YFP::mTalin (Fig. 6D, d) demonstrates mEosFP compatibility with GFP and YFP, respectively. In order to assess whether non photo-converted mEosFP can be used safely with a RFP the mEosFP-PTS1 was co-visualized with RFP-ER using 488 nm and 543 nm lasers (Fig. 6E). Simultaneous visualization of both probes demonstrated that the green form is stable and not prone to spontaneous photo-conversion. We then tested whether CFP, excited by the 458 nm laser could also be co-visualized with mEosFP. Both the green mEosFP-PTS1 and its red form could be visualized with CFP-labeled actin filaments. Co-visualization with CFP confirmed that mEosFP does not get photo-converted using the 458 nm laser and once again attested to the stability of both forms of mEosFP under simultaneous illumination by 458 and 543 nm lasers (Fig. 6F).

Conclusions
The observations presented here demonstrate that monomeric EosFP based probes retain all the qualities of single colored fluorescent proteins while providing the additional capability of photoconversion. The major strength of mEosFP probes lies in their ability to create color-based differentiation within and between organelle populations and membranes. Both green and red fluorescent forms of mEosFP are stable and thus provide the highly desirable intracellular controls during prolonged live imaging. These ready to use probes greatly enhance spatiotemporal precision during live imaging of plant cells and as demonstrated can be used to label single cells, small regions within a cell, track single organelles, and allow an analysis of membrane and cytoskeletal dynamics. All mEosFP based probes are amenable to differential colour quantification methods. As demonstrated by the discovery of tubular PI3P enriched compartments the new probes possess tremendous potential for further discovery within the plant cell.

Materials and Methods

Molecular methods

Table 1 (supplementary information) provides a list of PCR primers used for constructing different mEosFP fusion constructs. All PCR products were cloned into pGEM-T-easy vector before excising relevant fragments and inserting them into an intermediate vector carrying a pCaMV-35S promoter and a nos-terminator sequence. The binary pCambia 1300 base vector (http://www.cambia.org.au) to which a pCaMV-35S promoter and a nos-terminator sequence had been added was used for generating plant transformation competent mEosFP-cytosol, mEosFP-PTS1, p35S-CX::mEosFP, LIFEACT::mEosFP and mEosFP::MBD-MAP4. An additional version of mEosFP-cytosolic was driven by a GLABRA2 promoter, which is strongly active in trichomes (Szymanski et al., 1998). For the mito-mEosFP construct Eos was PCR amplified from a pcDNA3.1 clone (Reidl et al. 2008) using relevant primers (supplementary Table 1). The PCR product was purified and digested with SpeI and SacI. The backbone for this construct was pBINmGFP5-atpase (Logan and Leaver, 2000), cut with SpeI and SacI to remove mGFP5. The 5′-SpeI-Eos-3′-SacI was then ligated in to replace mGFP5. Standard molecular biology protocols were followed (Sambrook et al., 1989).
Expression in plant cells

Transient expression of different mEosFP probes was carried out in onion epidermal cell and 8-12 days old Arabidopsis seedlings. Gold-particle coating with DNA and bombardment using a biolistic particle delivery system (Bio-Rad PDS-1000/He; http://www.bio-rad.com/) was carried out following the manufacturer’s instructions. mEosFP expression was assessed between 16 and 20 h after biolistic particle bombardment. For mito-mEosFP the transient expression was carried out using agro-infiltration (strain GV3101 at 0.1 OD600) of tobacco leaves according to Sparkes et al., (2006). The images of mito-mEosFP expression were taken 6 days after infiltration.

Stable transgenic lines for CX::mEosFP, mEosFP-PTS1, mEosFP-cytosol, LIFEACT::mEosFP and mEosFP::MBD-MAP4 were generated using Agrobacterium tumefaciens (strain GV3101) mediated floral dip transformation method (Clough and Bent, 1998). Seeds were grown on 1% agar-gelled Murashige and Skoog (1962) medium, supplemented with 3% sucrose and with pH adjusted to 5.8. Plants were grown in petri dishes in a growth chamber maintained at 21± 2º C, and a 16/8 hour light/dark regime using cool white light at ca. 80-100 μmol m⁻² sec⁻¹.

Microscopy and drug treatments

For live imaging, plant tissue and seedlings were placed in a depression slide in distilled H2O under a glass coverslip. Four different microscope systems were used:

System 1 consisted of an upright epi-fluorescent microscope (Nikon eclipse 80i) equipped with a 40x Nikon Plan Apochromat lens with numerical aperture 0.95 and a 12-bit 3CCD -color digital camera (Qicam fast 1394; Qimaging, http://www.adept.net.au/cameras/qimaging/) was used for epi-fluorescent microscopy. For this set up the chroma filter sets used were Endow GFP-LP filter set 41018 (exciter (Ex), HQ 470/40X; Dichr, Q495LP; emmitter (Em), HQ500LP), TRITC filter set 41002c (Ex, HQ545/30X; Dichr, Q570LP; Em, HQ 620/60) and the DAPI/Hoechst/AMCA filter set 31000V2 (Ex 350/50X;Central dichroic LP 400; Em 460/50). Photo-conversion was performed manually using epi-fluorescent lighting through the DAPI filter cube. Images were captured and processed using SIMPLE PCI software (Compix Inc., http://www.cimaging.net).
System 2 consisted of a Leica TCS-SP5 confocal laser-scanning microscope equipped with a 488 nm Ar laser and a 543 nm HeNe laser. The epi-fluorescent setup consisted of a Leica DM6000CS microscope equipped with a 40x water immersion lens (numerical aperture 0.80). Images were obtained in a 1024 x 512 pixel format in x/y/z and x/y/time dimensions, and processed using proprietary LEICA software. Unless stated otherwise the time lapse between x/y/time scans was maintained at 1.37 sec. Sequential images had a 1µm (z axis) distance between them for x/y/z mode acquisition. Fluorescent emission collection was at 490–510 nm for GFP, 500–522 nm for YFP, 570–620 nm for RFP and 626–763 nm for chlorophyll. For visualizing CFP-mTalin (Fig. 6E) the probe was excited using 458 nm laser and emission collected between 493 and 510 nm (for CFP); between 511 and 540 nm for green and 568 to 600 nm for red forms, respectively, of mEosFP.

Photo-conversion was performed manually by controlling the diaphragm. The diaphragm on the Leica DM6000CS microscope was modified to achieve 50 and 100 µm apertures. Closing down the iris, or moving the stage so that only a small part of the cell was exposed to the beam, achieved photo-conversion of an organelle subpopulation. Epi-fluorescent lighting was through a D filter cube (Leica. UV/violet; ex BP 355-425/dichroic 455/ em LP 470 nm) and a 40x water immersion lens. For imaging the photo-converted probe simultaneously with non-converted FP the 488 nm laser and the 543 nm HeNe lasers were used at approximately 10% and 80% power, respectively. Fluorescence bleed through was minimized by adjusting the pinhole and PMTs and confirmed through sequential laser scans. However, for most visualization both Ar and HeNe lasers were used simultaneously.

System 3 comprised of a Leica TCS-SP5 confocal laser scanning microscope and multi photon imaging setup on an upright LEICA 6000B microscope with 63x (numerical aperture 0.90) water immersion lens. This system has a Radius 50 mW 405 nm laser in addition to the Ar and HeNe lasers. This set up was used only for checking probe conversion and utilized Leica proprietary FRAP software for controlling 405 nm, 488 nm and 543 nm lasers. It was not used routinely in the experiments presented here.
System 4 was used specifically for observing the mito-mEosFP infiltrated tobacco leaves and consisted of a Zeiss Axioimager-Z1 microscope with light from a HXP120 mercury lamp, an apochromat 63x oil immersion lens (NA=1.4), a Zeiss 38HE GFP cube (ex 470/40 /dichroic 495 / em 525/50) and a Zeiss 43HE Red cube (ex 550/25/ dichroic 570 / em 605/70). A Zeiss-49 (DAPI/Hoechst ex 365/50/dichroic 395/ em 445/50) filter was used for photoconversion.

For drug treatments both Latrunculin B and BDM were purchased from Molecular Probes (Invitrogen), dissolved in 30% DMSO and used at concentrations shown (Fig. 4). Plants used in control experiment depicted in Fig. 4 were placed in water containing an equivalent amount of DMSO.

Post-acquisition image processing and quantification

All images were cropped and processed for brightness/contrast as complete montages using Adobe Photoshop CS3 (http://www.adobe.com). The layer function in Photoshop was used to introduce text, regions of interest and color overlays. Green and red images for mito-mEosFP (Fig.6 a -inset) were merged post acquisition using NIH Image-J. Images acquired using the Leica confocal microscope were processed directly using the proprietary fluorescence intensity quantification tools in various regions of interest. Alternatively the histogram analysis tool that provides RGB data on a 8 bit value scale of 0 to 255 (Cowlishaw, 1985), Interactive 3D surface plot v2.22 (http://rsbweb.nih.gov/ij/plugins/surface-plot-3d.html) which uses image luminance for plot height, Colour inspector 3D v-2 which shows colour distribution within a 3-dimensional color space and allows color-cell frequency to be presented in histograms (http://www.f4.fhtw-berlin.de/~barthel/ImageJ/ColorInspector/help.htm), and the RGB profiler (http://rsbweb.nih.gov/ij/plugins/rgb-profiler.html) plug-ins from NIH ImageJ-1.40g (http://rsbweb.nih.gov/ij/) were used. Colour quantification (eg. colour bar in Fig. 1 C) followed the ICC compliant Adobe Photoshop colour coding as described (Schenkel et al., 2008).
All experiments reported here were replicated at least four times. Where applicable speed measurements are provided as mean value ± standard error followed by the total number (n) of observed cells/organelles.

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References:


Schenkel M, Sinclair AM, Johnstone D, Bewley JD, Mathur J (2008) Visualizing the actin cytoskeleton in living plant cells using a photo-convertible mEos::FABD-mTn fluorescent fusion protein. *Plant Methods* **4**: 21


Figure Legend

**Figure 1. Visualization of green and red forms of mEosFP distinct from chlorophyll fluorescence (false colored blue) in transgenic Arabidopsis plants expressing mEosFP-cytosolic.**

A-D. Differential labeling of a single celled trichome at an early developmental stage using non-targeted mEosFP-cytosolic expressed under a trichome specific GLABRA2 promoter. The inset RGB fluorescence intensity line traces in each panel depict the pre- and post- photo-conversion values of red, green and blue per pixel along the diagonal depicted in panel B. The RGB values are calculated on a standard 8-bit scale of 0 to 255 stretching across the ROI. Note the residual green fluorescence of mEosFP in panel C.

E. Mature trichomes on a transgenic Arabidopsis leaf visualized 48 hours after mEosFP-cytosolic had been photoconverted in the central trichome cell (arrowhead) show the stability of the red form of mEosFP following its photo-conversion. Note that simultaneous visualization of both green and red forms provides the comparative controls within a single scanned image.

F. Two pairs of guard cells developing in an aberrant pattern on a transgenic mEosFP-cytosolic plant demonstrate the major colors that can be obtained following epi-fluorescent photoconversion followed by simultaneous visualization. The top guard cell (red) contains fully photo-converted mEosFP-cytosolic (region of illumination ringed by broken line); a partially photo-converted nucleus appears yellow while cytoplasmic aggregates in the lower pair of guard cells retain the un-converted G-mEosFP form. The inset on the left inset depicts colour luminance along the marked region of interest (ROI), which is helpful in quantifying and interpreting color overlap following photo-conversion.

G. A nucleus in a hypocotyl epidermal cell of a light grown seedling exhibits the R-mEosFP form without an intentional photo-conversion and suggests that the red form might accumulate within the nucleus.

All images were taken using microscopy system 2. Size bars (µm): A – E = 25; F = 10; G = 5.
Figure 2. mEosFP-based probes targeted to different membrane compartments.

A. A region lying between two onion epidermal cells transiently expressing mEosFP::PIP1 probe shows the non-converted (green) and the photo-converted (red) labeling of tubular-vesicular membranes. Arrowhead points to the plasma membrane. (also see Supplementary Fig. 1)

B. Membrane tubules and multi-lamellar vesicles (arrowhead) in an mEosFP::PIP1 expressing cell. Vesicles are motile and the red (photo-converted) mix readily with non-converted (green) vesicles within minutes. The merged image acquired from both green and red channels is a single scan along XY axis. Due to rapid motility of vesicles and tubules separate pre- and post-conversion images from green and red channels do not exhibit the vesicles observed here and can only inform about the non-visibility of the probe in the red channel prior to its photo-conversion.

C. D. An onion epidermal cell transiently expressing mEosFP::α−TIP1 probe shows vacuolar membranes being highlighted. The photo-converted portion of membrane (arrowheads in C, D) delimiting a mini-vacuole appears red and shows a regular spherical shape (C) that changes into an ellipsoid form in a subsequent scan (D). The merged images are sufficient to show dynamic changes in highlighted compartment like conventional FPs but through sequential time-lapse imaging have the potential to inform about membrane dynamics within the vacuole compartment.

E. Differential labeling of various PI (3)P enriched vesicles following transient expression of mEosFP::2xFYVE in an onion epidermal cell. Based on their size the smaller vesicles (eg. boxed in area) qualify as endosomes while the larger vesicles (eg. *) are considered pre-vacuoles and vacuoles. The number of endosomes is fairly representative of onion cells exhibiting active cytoplasmic streaming. A number of pre-vacuoles (eg. arrowhead and *) display internalized vesicles. Full photoconversion labels vesicles red whereas non-converted vesicles remain green. Partial conversion results in labeling hues ranging from green to red.

F. A single mEosFP::2xFYVE labeled vesicle (arrowhead) illuminated with a 3 second pulse of violet blue light exhibits internalized vesicles of different colors. This suggests
that sequential photo-conversion pulses may be used to determine the relative differences in protein content or membrane labeling between vesicles. Arrow points to an unconverted vesicle used as control.

G. Rapid membrane fusion observed along a line of contact between photoconverted and non-photo-converted vesicles (arrowhead) achieved through transient salt induced plasmolysis suggests usefulness of mEosFP::2xFYVE probe for understanding homotypic vesicle fusion.

H. A PEG treated cell exhibiting aggregation of numerous small mEos::2xFYVE vesicles around the larger ones. This state is maintained until PEG is removed, when rapid vesicle fusion occurs.

All images were acquired using microscopy system 2. Size bars (µm): A-D, F-H = 5;E = 1.

Figure 3. Tubular projections from mEosFP::2xFYVE highlighted PI(3)P enriched vesicles.

A. An onion epidermal cell transiently expressing mEosFP::2xFYVE exhibits differentially coloured vesicles and associated tubules of varying diameters range from very thin (small arrows) to thick (large arrowheads) after a 5 second exposure to violet blue light. Fused vesicles in the bottom right ‘*’ extend multiple, thin tubules from their surface. Facing arrowheads depict a junction where two tubules extended from different vesicles appear to meet. Photoconverted protein does not extend beyond the junction suggesting that despite their proximity the tubules maintain closed ends.

B. A single vesicle exhibiting a tubule and an oscillatory movement resulting in the zigzag shape of the tail.

C. Six frames from a time-lapse sequence taken over 24 seconds shows a single photoconverted tubule and its contortions. Note that the tubule appears to snare a vesicle. (Supplementary information. Movie 1).

D. A single tubule that has ensnared many vesicles within a cup-shaped structure.
All images acquired using microscopy setup 2. Size bars - A = 5 µm; B-D = 2.5 µm.

**Figure 4.** CX::mEosFP demonstrates potential for estimating membrane flow through analysis of initial-colour recovery after photoconversion using microscopy system 2.

A. A cell from a transgenic *Arabidopsis* seedling expressing CX::mEosFP shows unconverted (green-upper portion) and photo-converted (red-bottom) labeling of cortical ER membranes.

B. Locally photoconverted CX::mEosFP (broken line circle) in a cell reveals a quantifiable color gradient (depicted in the inset) due to the rapid mobility and intermixing of red and green highlighted ER membranes. This observation formed the basis for colour tracking shown in panels C to E.

C. Time lapse sequence over 40 seconds shows the initial (non-converted) green state of CX::mEosFP (panel 1 at 0 second). Photo-conversion carried out using a circular beam from a violet blue D filter for 5 seconds made a subcellular region fluoresce red (panel 2 at 5 seconds). Subsequent recovery of the green colour in the delineated region of interest lying along the diagonal line within the photo-converted area (ROI-box in panel 1 and hatched line in subsequent time panels) is shown taking place over the next 7 steps.

D. Line traces of diagonals in successive panels from ‘C’ subjected to colour quantification using a standard 0 to 255 RGB scale depict the change in green and red levels and the nearly complete recovery of green fluorescence over time (eg. time point 0 compared to 40 seconds).

E. A comparison of CX::mEosFP movement over 70 seconds in untreated control cells and cells treated with 0.1, 1.0 µM Latrunculin B and 10 mM BDM following a photoconversion step of 5 seconds. Green / red ratios (averaged from three separate experiments) plotted against time demonstrate that the relative green fluorescence recovery time varies with cellular conditions. The method can therefore be applied to analyze and compare protein and membrane mobility in cells.

Size bars = 10 µm
Figure 5. mEosFP based probes for cytoskeletal elements.

A, B. LIFEACT::mEosFP represents the smallest live-imaging probe for F-actin. Actin filaments in transgenic Arabidopsis plants are efficiently highlighted in green (ROI in A) before and in red (B) after photoconversion. Chloroplasts (chl- false colored blue) are co-visualized with both forms of mEosFP.

C, D. Transgenic Arabidopsis plants expressing mEosFP::MBD-MAP4 highlight cortical microtubules (C), which have been photoconverted locally (D) in a specific region of interest (ROI shown in C). Co-visualized chloroplasts are depicted in blue.

E. Tracking changes in cytoskeletal elements is possible using mEosFP labeling. Shown in E1 to E4 are images taken 10 seconds apart to show a change in F-actin organization as filaments (E1) bundle together (E4). The directional dispersal of photo-activated red form of mEosFP is apparent between the four images and provides a means of assessing F-actin polarity in living cells. An independent positional marker on the sequential images (eg. arrows in panels E1 to E4) may minimize interpretational errors arising from a cytoplasmic streaming induced drift in the actin cable.

F. A post-image acquisition 3-D surface plot (Image J) of the ROI depicted in E1 (and subsequent images) provides quantization of red and green fluorescence values existing in an image on a standard RGB scale (values between 0- 255). Using these values a R/G ratio of 1 in the acquired image suggests equal green and red molecules while ratios higher than 1 suggest increased red levels. F1 to F4 depicts the shift in red fluorescent molecules towards the right side of the time-lapse images. Alterations in the R/G ratio (arrowheads in F4) suggest that new, un-photoconverted monomers are being added from one end (left side of images) and therefore cause the photo-converted F-actin region to shift towards the right. As indicated by the arrowheads (F4) the net fast growing, barbed ends of the actin filaments lie on the left.

All images were acquired using microscope system 2. Size bars: A, B, E, 5 µm; C, D = 20 µm.
Figure 6. Visualizing motile organelles using mEosFP alone and in combination with other FPs.

A. a. B. b. Mito-mEosFP transiently expressed in tobacco leaves by means of agro-infiltration efficiently targets to mitochondria (A) and is near fully photoconverted (b) after a 30 second exposure to 365/50 nm wavelength. Microscope system 4 was used for acquiring these images. Note that while pre-conversion fluorescence is barely detectable in the red channel (a) following photo-conversion, it is not picked up in the green channel (B). The exposure times for image (A) and (B) are identical, as are those for images (a) and (b). The inset placed in (a) is a merged image of the green and red channels post-conversion using identical exposure times and demonstrates the slight shift and resultant artifact (arrowheads) that occurred during the time-lapse between capture of the two sequential images which could be mis-interpreted as an absence of co-localization.

C. c. Golgi bodies highlighted by mEosFP::GONST1 and F-Actin (highlighted using GFP::mTalin) co-visualized before (C) and after photoconversion (c). The rapid motility of Golgi bodies does not allow direct comparisons to be drawn between pre and post conversion images in separate channels and therefore merged images acquired in both channels are presented. The inset 3D-surface plot clearly shows quantifiable changes in the color of Golgi bodies from their pre- to the post- photo conversion state. (See Supplementary Fig. 2 for more detail).

D. d. Peroxisomes labeled with mEosFP-PTS1 and F-actin labeled using YFP::mTalin co-visualized before (D) and after photoconversion (d).

E. Peroxisomes labeled with mEosFP-PTS1 can be clearly discriminated (arrowhead) from RFP-labeled ER during prolonged co-visualization using 488 and 543 nm lasers. Unintended photoconversion of mEosFP does not occur.

F. Both the non-photoconverted (arrowhead, green) and photo-converted (circle, red) forms of mEosFP-PTS1 co-visualized with CFP::mTalin targeted to F-actin. Photoconversion was carried out separately since the 458 nm Ar laser line does not cause mEosFP to change colour.
Images C to F acquired using microscope system 2. Size bars: 5 µm.
Table 1. Photo-convertible mEosFP-based probes targeted to different subcellular compartments

<table>
<thead>
<tr>
<th>Name of Probe</th>
<th>Target</th>
<th>Sequence used for targeting / Base reference</th>
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</thead>
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<tr>
<td>mEosFP-cytosolic</td>
<td>cytosol</td>
<td>†Non-targeted monomeric EosFP</td>
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<td>At1g73190: CDS alpha tonoplast intrinsic protein / Hunter et al. 2007</td>
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<td>†At5g61790: membrane targeting sequence of calnexin 1/ Runions et al. 2006</td>
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<td>‡2X-FYVE domain from mouse HGF-regulated tyrosine kinase substrate protein / Voigt et al. 2005</td>
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<td>At2g13650: CDS GONST 1/ Baldwin et al. 2001.</td>
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<td>mEosFP::PTS1</td>
<td>peroxisome matrix</td>
<td>‡C-terminal tripeptide ‘SKL’ (PTS1)/ Mathur et al. 2002; Sinclair et al. 2009</td>
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<td>microtubules</td>
<td>†Microtubule binding domain mammalian MAP-4 / Marc et al. 1998</td>
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<td>†17 aa peptide from Yeast Abp140p / Reidel et al. 2008</td>
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<tr>
<td>mEosFP::FABD-mTn</td>
<td>F-actin</td>
<td>‡F-actin binding domain of mammalian Talin / Kost et al. 1998; Schenkel et al. 2008</td>
</tr>
</tbody>
</table>

1 Transgenic lines created in *Arabidopsis thaliana*

2 Previously reported on basis of transient assays.