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Methods for studying movement of molecules within cilia

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Summary

The assembly of cilia and eukaryotic flagella (interchangeable terms) requires the import of numerous proteins from the cell body into the growing organelle. Proteins move into and inside cilia by diffusion and by motor-based intraflagellar transport (IFT). Many aspects of ciliary protein transport such as the distribution of unloading sites and the frequency of transport can be analyzed using direct *in vivo* imaging of fluorescently tagged proteins. Here, we will describe how to use total internal reflection fluorescence microscopy (TIRFM) to analyze protein transport in flagella using in the unicellular alga *Chlamydomonas reinhardtii*, a widely used model for cilia and cilia-related disease.

Keywords

cilia; flagella; green fluorescent protein; intraflagellar Transport (IFT); total internal reflection fluorescence microscopy (TIRFM)

1. Introduction

Fluorescence-based microscopy has revolutionized the analysis of proteins in living cells and organisms. Genetically encoded fluorescence proteins (FP) such as green fluorescence protein (GFP) can be fused to virtually any protein permitting one to track its localization and changes thereof *in vivo*. However, often noise in the form of autofluorescence or out-of-focus signal from other regions of the cell will reduce the image quality and prevent the imaging of single proteins. These hurdles can be overcome by techniques that will excite fluorophores only within a limited focal plane or by imaging thin portions of the cells such as cell projections. Total internal reflection fluorescence (TIRF) imaging of cilia, thread-like cell extensions of several μm length, combines both of these features: Depending on the illumination angle, TIRF has a reach of 30–300 nm from the surface of the cover glass and thus is a useful tool to image objects close to the cover glass. Cilia and flagella have a diameter of approximately 200 nm, and, when cilia are appressed to cover glass, fluorophores in the entire ciliary volume can be excited and imaged by TIRFM with a high signal-to-noise ratio. When optimally positioned, the cell bodies will remain out of range of the TIRF excitation depth avoiding autofluorescence, out-of-plane signal, phototoxicity, and

photobleaching. TIRF imaging has been applied to study protein transport in cilia of various systems including *Tetrahymena thermophila* and mammalian cells [1,2]. The flagella of *Chlamydomonas reinhardtii* are excellently suited to study protein transport by TIRFM because the cells naturally adhere via their two 12- μ m long flagella to smooth surfaces such as cover glass [3]. This which will immobilize the cells and the flagella will be positioned within the TIRF excitation range while the cell bodies remain out of reach. Further, numerous *C. reinhardtii* mutants for IFT and other ciliary proteins are available; this collection includes conditional mutants in which IFT can be switched-off by a temperature shift [4]. Null mutants can be rescued with FP-tagged versions of the affected genes effectively replacing the endogenous protein with a FP-tagged version [5]. Moreover, flagellar assembly can be easily experimentally induced in *C. reinhardtii* allowing for an analysis of flagellar growth, genetically distinct cells can be fused by mating to study flagellar repair and turnover, and flagella can be isolated in bulk to determine their biochemical composition and changes thereof as they are caused by defects in ciliary protein transport in an unbiased manner. Here, we will provide step-by-step instructions on how to image protein transport in the flagella of *C. reinhardtii* using TIRF microscopy.

2. Materials

2.1. Preparing strains expressing fluorescent protein-tagged ciliary proteins

- 1 PCR machine, DNA polymerase with proof reading activity, restriction enzymes, agarose electrophoresis, cell electroporator.
- 2 Templates for fluorescent proteins (table 1 and <http://chlamycollection.org/>)
- 2 M-medium (<http://chlamycollection.org/m-or-minimal-media/>); TAP-40mM sucrose (sterile filtered; <http://www.chlamy.org/TAP.html>).
- 3 Culture plates with selective media, 96- and 24 well plates, toothpicks.
- 4 Microscope, Western blotting equipment, rabbit polyclonal antibody to GFP (A-11122 life technologies), antibodies specific for the target protein.

2.2. Sample preparation

1. 20×60 mm No. 1.5 and 22×22 mm cover glass.
2. Syringe with petroleum jelly or silicone vacuum grease.
3. Immobilization buffer (10 mM Hepes, 5 mM EGTA, pH 7.2).

2.3. *In vivo* microscopy

1. TIRF microscope with a 60x or 100x NA 1.49 TIRF objective (NA should be at least 1.45).
2. Lasers of the desired wave lengths, e.g., 488 nm for GFP, sfGFP, and mNeonGreen and 565nm for mCherry, tdTomato, etc. Corresponding excitation and emission filters.
3. Single photon EMCCD camera, e.g., Andor iXon 897 or similar.

4. Imaging software, e.g., µManager (https://micro-manager.org/wiki/Download_Micro-Manager_Latest_Release; this site will soon move).

2.4. Analyzing protein transport during flagella regeneration and repair

1. Flagella amputation by pH-shock and regeneration: Stirrer, pH meter, 500 mM acetic acid, 250 mM KOH, fresh M-medium, centrifuge, shaker.
2. Long-zero cells (cells with only one flagellum): 1-ml syringe, 26G½ needle.
3. Mating: Strains of opposite mating type (+ and –), M-N medium (<http://chlamycollection.org/m-or-minimal-media/>), 20% M-N medium supplemented with 10 mM Hepes pH7.2, 150 mM dibutyryl(db)-cAMP (Sigma) in water, centrifuge, shaker.

2.5. Analysis of imaging data

1. ImageJ (<http://rsbweb.nih.gov/ij/download.html>) or Fiji (<http://fiji.sc/Fiji>). For ImageJ, you will need to download the Multiple Kymograph plugin (http://www.embl.de/eamnet/html/body_kymograph.html); the LOCI Bioformats plugin (<http://loci.wisc.edu/software/bio-formats>) will allow you to open files from various imaging softwares in ImageJ.
2. Microsoft Office Excel

3. Methods

3.1. Construction and transformation of expression vectors for FP-tagging of flagellar proteins

C. reinhardtii allows stable nuclear transformation of endogenous and heterogeneous genes [6]. After PCR amplification using either total genomic DNA, total cDNA, or plasmid DNA as a template, the gene of interest (GOI) is fused to GFP or other FPs many of which have been codon-adapted and positively tested in *C. reinhardtii* (Table 1, see Note 1). Complete synthesis of genes is an affordable alternative, in particular for small genes. In addition to N- or C-terminal fusions, FPs have been also inserted into genes and successfully expressed (Fig. 1). In general, the presence of the GOI and the selectable marker gene on the same plasmid will increase the number of positive transformants. For best results, the construct should be expressed in a mutant null for the corresponding endogenous gene. The FP-tagged GOI should rescue the mutant phenotype verifying the functionality of the fusion protein; it is then reasonable to assume that the fusion protein mirrors the distribution and behavior of the endogenous protein. Expression in wild-type strains, however, can be also achieved in particular using the recently introduced polycistronic vectors, in which transgene expression is linked to antibiotic resistance using a viral 2A sequence encoding a self-cleaving peptide

¹Among the various green FPs, mNeonGreen [12] is currently the best choice: it folds quickly and is considerably brighter (2.6 ×) than EGFP (Table 1). The maturation time of a FP can be important, e.g., when protein transport during ciliary regeneration is studied: Freshly translated proteins might be transported into cilia before the FP is able to exhibit fluorescence. The red FP mCherry, while significantly dimmer and less stable than GFP, is a good compromise between maturation time and brightness. Also, note that the folding/maturation of many FPs is accelerated at higher temperatures.

[7]. The presence of both the endogenous wild-type and FP-tagged transgenic protein warrants special caution when interpreting data.

1. Various strategies have been successfully used to express IFT and other ciliary proteins as FP-fusion proteins in *C. reinhardtii*; some of the vectors are depicted in Fig. 1.
2. If possible, linearize the expression vector or cut out the cassette containing the selectable marker and GOI. Purify the DNA by agarose gel electrophoresis and gel elution. Verify fragment size, DNA concentration, and purity by electrophoresis of an aliquot of the eluate (see Note 2).
3. Wash cells in TAP-40mM sucrose and concentrate cells to $\sim 1 \times 10^8$ cells/ml. Pipette 80 μ l of cells into pre-chilled electroporation cuvettes and add ~ 100 – 800 ng of DNA. Alternatively, premix cells and DNA before transferring the mix into the cuvettes. Always prepare a non-DNA control for each strain to be transformed.
4. Transform cells by electroporation. We use a BTX Electro Cell Manipulator 600 with the following settings: Capacitance & Resistance: 500V, Resistance Timing: R1 (12 Ω), Capacitance Timing: 1000 μ F, Set Charging Voltage: 200V. Adjust volume and settings for other cuvette sizes and electroporators, respectively (see Note 3).
5. Transfer cells to 10 ml of TAP+sucrose and incubate overnight in light with agitation. Concentrate cells to ~ 500 μ l, mix with ~ 2 ml of liquid TAP-0.5% agarose at $\sim 40^\circ\text{C}$, and pour mix onto ambient-temperature TAP plates with selective medium. Follow a protocol describing the plating of phages with top agarose. Incubate plates in constant light until colonies are clearly visible (see Note 4).
6. Using sterile toothpicks, transfer colonies into ~ 150 μ l of M-medium in a 96-well plate. If possible, check for the rescue of flagellar growth, motility, or phototaxis phenotypes by light microscopy; this should be done ~ 2 hours after picking the colonies and then repeatedly over the next couple of days to ensure consistency of the phenotype (see Note 5).

²Using linearized vectors or purified fragments will increase the transformation rate (colonies/ μ g of DNA). Instead of gel-purified DNA, alcohol-precipitated DNA works as well. The presence of enzymes will affect the transformation rate of electroporation, but not that of the glass-bead method [6].

³In contrast to glass bead transformation [6], which mostly results in a high co-transformation rates ($\sim 90\%$) even when the selectable marker gene and GOI are on separate plasmids, transformation by electroporation performs better when both genes are on the same plasmid. For co-transformation, a ratio of at least 1:5 between the selectable marker plasmid and the GOI plasmid should be used. Because smaller plasmids enter cells easier, the ratio should be increased when the GOI plasmid exceeds the selectable marker plasmid in size. Too much DNA will decrease transformation rates for electroporation and will increase the share of transformants with more than one GOI plasmid integrated into the genome; this can lead, e.g., to rapid silencing of the construct. Also, be aware that DNA inserts randomly into the *Chlamydomonas* genome and thus can cause new mutations. Compare several positive transformants for consistency of phenotype.

⁴Transformants should appear after 6–8 days for aph7⁺/hygromycin and after 8–11 days for BLE/zeocin. Generally, transformants should be absent from the no-DNA control plates but some flagella-less mutants display an increased resistance against antibiotics.

⁵Rescue rates vary greatly from $>60\%$ – $<1\%$. pBR vectors allow for expression of tagged genes in wild-type cells; expression of the GOI is typically observed in $>50\%$ of the transformants [7]. Due to the linkage of antibiotic resistance to GOI expression, transformants expressing the GOI often grow faster after transfer to the 96-well plates.

7. Screen transformants for expression of the GOI-FP fusion using TIRF microscopy of living cells and/or Western blotting and/or immunofluorescence with anti-GFP or a protein-specific antibody (see Note 6).

3.2. Sample preparation

C. reinhardtii swims by means of two motile 9+2 flagella. The observation of protein transport and IFT in cilia requires immobilization of the cells. This can be achieved by gluing the cells to the cover glass using, e.g., poly-L-lysine, by using immotile *paralyzed flagella* (*pf*) mutants strains, or by agarose embedding of the cells. Best results, however, are obtained by allowing the cells to naturally adhere to the untreated cover glass via their two flagella. Gliding motility [8] can be suppressed by removing calcium from the environment using EGTA (see Note 7).

1. Wash cells into fresh M-medium (see Note 8).
2. Apply a ring of vacuum grease or petroleum jelly with a diameter of ~15 mm onto a clean 24x60 mm cover glass; this serves as a spacer to avoid compression of cells and to seal the specimen.
3. Place ~5–11 µl of cell suspension onto the cover glass and allow cells to adhere for 1–5 min depending on cell density (see Note 9).
4. Apply an equal volume of immobilization buffer (~5–11 µl) onto a 22x22mm cover glass; use a similar pattern of drops as generated in Step 2. Lower the 22x22mm-coverslip with the drops hanging down onto the large coverslip to prepare the observation chamber. Close the chamber immediately before imaging of the cells. We use slides for up to 30 minutes.

3.3. In vivo imaging

Imaging proteins inside flagella has to overcome two problems: 1) signals are typically relatively weak because many ciliary proteins are transported in small clusters or as single proteins, and 2) the *C. reinhardtii* cell body is very autofluorescent due to its large chlorophyll-containing plastid. The latter can be surmounted by selective excitation of just the flagella [9]; the former will need a light-sensitive camera (see Note 10). TIRF microscopes are optimal for imaging proteins in flagella appressed to cover glass. TIRF illumination typically excites fluorophores in a ~30–300 nm region which is well below the

⁶The FP-fusion protein should be ~27 kDa larger than the target protein alone; expression from pBR vectors can lead to incomplete cleavage of the product resulting in a shBLE-FP-target protein fusion that is ~52 kDa larger than the unmodified protein. Use a strain with verified expression of a FP-fusion protein as a control for Western blotting and especially to adjust the TIRF microscope.

⁷It remains to be determined to which extent the removal of external calcium affects protein transport in cilia. We compared the velocity of IFT and the transport frequency of the axonemal protein DRC4 in samples prepared with and without the EGTA-containing immobilization buffer without noting a difference. Transport of the transmembrane protein FMG-1, however, appears to require external calcium [8].

⁸This is optional for cells in mid-log growth phase but required for dense cultures because the cells from such cultures tend to not adhere properly to cover glass presumably due to the accumulation of secreted material in the culture medium.

⁹For low density cultures (~10⁶ cells/ml) apply one drop into the middle of the ring; for dense suspensions apply several small drops amounting to ~5–11 µl; this will make it easier to find a region in which the cells are nicely spaced (Fig. 2). High cell densities negatively affect cell immobilization.

¹⁰FP-tagged IFT proteins are easy to visualize by various microscopic techniques including standard epifluorescence. Note that cargoes are typically much less abundant in an IFT train than the IFT proteins: a train is composed of dozens of IFT particles, only a few of which might carry the cargo of interest.

optical section thickness of most confocal fluorescence microscopes; signal intensity decreases exponentially with distance from the cover glass effectively eliminating fluorescence from the cell body. We use a home-build through-the-objective TIRF system, which has been previously described in detail [3]; commercially available TIRF systems will work just as well.

1. Place the chamber onto the microscope with the large 24×60 mm coverslip facing the objective; center the spot of adhered cells into the middle of the objective.
2. Using the microscope's standard light source focus onto the plane in which the flagella are attached to the cover glass. Ensure that cells attached to the larger bottom cover glass are in focus.
3. Turn-off standard light and switch to TIRF illumination. FP-tagged proteins in the flagella should be visible (see Note 11).
4. Frame rates of 5–10 fps are sufficient to document protein transport by IFT; higher frame rates (20 fps) are recommended to analyze the diffusional behavior of proteins inside cilia.

3.4. Imaging of protein transport during flagellar growth and repair

The transport frequency of tubulin and other axonemal proteins is upregulated during flagellar growth making it easier to observe transport events [10,11]. Flagella can be easily removed from cells by a pH shock and cells will largely synchronously regenerate flagella within ~80 minutes. Flagella can be also amputated by mechanical shear. Mating of a cell expressing a FP-tagged ciliary protein to a mutant strain defective in the corresponding endogenous gene allows for the analysis of protein transport during flagellar repair (see Note 12). Because the mutant-derived flagella of such zygotic fusion cells initially lack FP-tagged proteins, protein transport in flagella can be imaged with remarkable clarity (Fig. 3).

3.4.1 Flagellar amputation by pH shock

- 1 Place about 60 ml of cell culture into a beaker of appropriate size (~100 ml total volume), add a stir bar, and stir rapidly. Monitor pH using an electrode (see Note 13).
- 2 Using a transfer pipet, rapidly add ~1.5 ml of acetic acid (0.5 M) until pH reaches ~4.3. Monitor pH while adding the acetic acid. Do not overshoot 4.0. Wait for 45 seconds.

¹¹Laser strength should be as low as possible to avoid photobleaching. Make sure to adjust TIRF illumination to minimize stray light reaching the cell body: the autofluorescence of the cell body should be hardly visible while tagged proteins in the flagella are bright. This might require adjustment of the TIRF angle and centering of the TIRF spot. Often, TIRF illumination is only realized for a portion of the field of view.

¹²Many motility mutants of *C. reinhardtii* can be rescued by dikaryon complementation. After fusion of the mutant and the wild-type cell, wild-type protein present in the now shared cytoplasm will enter the mutant-derived flagella. Repair typically occurs within 20–60 minutes. However, several mutant phenotypes cannot be rescued in dikaryons or only after the old mutant flagella have been amputated. These include *pf18* and *sup-pf3* [13].

¹³Older cultures often have lowered the pH of the medium to ~6 and are best washed into fresh M-medium prior to deflagellation.

- 3 Add KOH (0.25 M) until pH reaches 6.5 – 7. Do not overshoot pH7, the pH will initially increase slowly, but will rise rapidly once the pH is above 6.
- 4 Immediately place cells in fresh M-medium (by centrifugation at 900×g for 3 minutes at RT) and store on ice.
- 5 Verify deflagellation by comparing cells before and after pH shock by light microscopy. For most strains more than 98% of the cells should have shed their flagella.
- 5 Allow an aliquot of cells to regenerate flagella in bright light at room temperature with agitation. After ~20 minutes, check cells for flagellar regeneration and image cells (see Note 14).

3.4.2 Generation of long-zero cells

1. Forcefully pass the cells for 3–8 times through the needle. Check for spinning cells using a microscope at low magnification. Spinning cells are likely to have lost one of their two flagella.
2. Start imaging after ~10 minutes. Long-zero cells will rapidly regrow the missing flagellum. Use a standard light source to locate long-short cells which account for ~1% of the total cells.

3.4.3 Generation of ciliary chimeras by mating

1. Spread cells on M-plate and incubate for 10 days in the culture room with a ~14:10 hour light:dark cycle at ~22 °C.
2. After 10 days, place the plates for 1 or 2 days in dim light in the culture room.
3. In the evening before the experiment, transfer cells from the plates into 10-ml of M-N medium in a small Erlenmeyer.
4. Incubate cells overnight in bright light with constant shaking at RT or somewhat warmer.
5. In the morning, mixing small aliquots of the cells to check for mating reaction by light microscopy. Well mating cells will form clumps.
6. If the mating is insufficient, place cells in ~5 ml 20% M-N plus 10 mM Hepes and continue to shake in bright light. Mating ability should peak after ~4 hours. Many mutant cells fail to form cilia of proper length when agitated on a shaker. Place such strains in bright light without agitation.
7. Once cell clumping is observed, mix an appropriate volume of the two mating strains. Start imaging after ~5 minutes and search for quadriflagellate zygotes

¹⁴TIRF imaging requires cells to adhere which works better once flagella exceed the length of 2–3 µm. Cells can be kept on ice for hours without impairing their ability to synchronously regenerate flagella; aliquots can be removed at various time points and incubated to room temperature to initiate flagella formation. Cell wall-deficient strains do not properly regenerate flagella; flagellar regeneration is also slower in many mutants as well as in some strains rescued with FP-tagged IFT proteins.

(see Note 15). Zygote formation can be triggered by adding db-cAMP (15 mM final concentration).

3.5. Analysis of transport velocity

The movement of proteins inside cilia can be described using various parameters such as mode of movement (diffusion or IFT), velocity, frequency, processivity (how long a cargo remains attached to IFT) etc. Unless one uses a trained particle tracking software, kymograms are the best way to determine these features. Kymograms are time-distance plots displaying the protein dynamics within cilia of an entire movie in a single image (Fig. 3).

1. Open movie file in ImageJ or Fiji.
2. Select the straight or segmented line tool by clicking on the icon.
3. Place a line running from the cell body to the tip of the cilium to be analyzed. Adjust line thickness by double click the line icon to adjust line width so that the line covers the entire width of the cilium.
4. Select the “Multiple Kymograph” tool under “Analysis” (Fiji) or “Plugins” (ImageJ). Choose “Linewidth” equals 1 and hit Okay.
5. Rotate the kymogram 90° counterclockwise. IFT-like trajectories should be oriented as shown in Fig. 3. Proteins moving anterogradely from the ciliary base to the tip should result in trajectories running from the bottom left to the top right, and vice versa for retrograde transport.
6. Switch to the Angle tool by clicking on the icon, use the tool to trace a trajectory on the kymogram from the bottom right to top left and measure the angle θ by pressing ‘CTRL M’.
7. Copy the data to Excel. Use the following equation to determine the velocity:

$$\text{Velocity } (\mu\text{m/s}) = \text{TAN}(\pi/180 \times \theta) \times \text{fps} / (\text{pixels}/\mu\text{m}); \text{fps} = \text{frames per second.}$$

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¹⁵Mating efficiency can vary greatly between strains and experiments (0 – 85% zygotes); some cell fusion can occur within 1 minute after mixing of the gametes but many strains require more time to form zygotes.

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Figure 1.

Chlamydomonas expression vectors for FP-tagging

Schematic presentation of various vector designs used to express ciliary proteins fused to fluorescent proteins (FP) in *C. reinhardtii*.

a) FP gene inserted in the protein coding region of the gene of interest (GOI); this technique was used, e.g., to express DRC4-GFP [17].

b) Expression vector for cDNAs based on the intron-free *FLA14* gene. The GOI can be inserted into a unique NheI site downstream of the ATG of the *FLA14* gene and upstream of

the gene encoding the FP. The vector has been used to express IFT20-mCherry and BBS4-GFP and rescue the corresponding mutants [16]. This vector will add the sequence MASLA to the N-terminus of the expressed protein.

c) Bicistronic pBR expression vector using the self-cleaving 2A sequence to express two proteins from one mRNA. The vector will add the residue P to the N-terminus of the protein following the 2A sequence. Also, cleavage fails for a significant portion of the encoded protein resulting in a fusion protein enlarged by an additional ~25 kDa. The vector has been successfully used to express alpha-tubulin fused to various FPs [7,10].

d) pBR-2xsfGFP vector for convenient expression of proteins fused either N- or C-terminally to sfGFP. The vector has been successfully used to express mNeon, mCherry, and sfGFP-tagged radial spoke proteins in *Chlamydomonas*.

e) Inverted pBR vector allowing for the expression of proteins with their original N-terminus and C-terminal fusion to FP (see Note 16). The vector has been successfully used to express IFT20 and phospholipase D with a C-terminal mNeonGreen tag..

¹⁶An unaltered N-terminus can be critical for protein function. Myristoylation, for example, occurs co-translationally on the second glycine and requires removal of the initial methionine by a special peptidase. This reaction is unlikely to work when residues are added to the N-terminus. Myristoylated proteins are enriched in cilia [14].

Figure 2.
Assembly of observation chambers for *in vivo* imaging of protein transport in *C. reinhardtii* flagella. See text for details.

Figure 3.

) Determining the velocity of IFT transport based on kymograms.

The kymogram depicts various phases of transport of the axonemal protein DRC4-GFP in an initially DRC4-deficient flagellum of a *pf2*×*pf2* DRC4-GFP zygote. Transport by IFT, unloading, diffusion, and docking (incorporation into the axoneme) can be observed. See text on how to calculate the velocity of particles from the angle θ of the IFT-like trajectories. Reprinted in modified form from Wren et al. 2013 [11].

Table 1

Fluorescence proteins successfully expressed in the cytoplasm of *C. reinhardtii*

color	name	max. excitation / emission (nm)	Brightness (% of EGFP)	Maturation (min)	stability	Monomer (m)/Dimer (d)	Codon usage	reference
green	CrEGFP	488/507	100	25	174	m	Cr	[15]
green	sfGFP	485/510	161	~6	157	m	Hs	[10]
green	mNeonGreen	506/517	276	<10	158	m	Cr	[10]
red	mCherry	587/610	47	15–40	96	m	Cr, Hs	[16,7]
Orange-red	tdTomato	554/581	283	~60	?	d	Cr	[7]
yellow	Venus	515/528	156	40	15	m	Cr	[7]
blue-green	mCerulean	433/475	79	?	36	m	Cr	[7]
blue	mTagBFP	402/457	98	50	34	m	Cr	[7]

Cr, *C. reinhardtii*; Hs, *Homo sapiens*. Stability: time in s to photobleach from 1,000 to 500 photons per s per molecule under arc-lamp illumination. Maturation: Time in min for fluorescence to obtain half-maximal value after exposure to oxygen (see Note 1). Sources: <http://www.my-whiteboard.com/properties-of-bright-and-photostable-monomeric-fluorescent-proteins/>; <http://nic.ucsf.edu/FPvisualization/>; <http://zeiss-campus.magnet.fsu.edu/articles/probes/anthozoapfs.html>; [http://www.weizmann.ac.il/plants/Milo/images/fluophoreMaturation140619Clean.pdf](http://nic.ucsf.edu/dokuwiki/doku.php?id=fluorescent_proteins)