CHAPTER 5

Targeting GFP to Organelles

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I. Introduction

The possibility of directly identifying intracellular organelles in living cells has a major relevance in cell biology studies. Indeed, not only can key events, such as organelle distribution and dynamics, be monitored, in a variety of physiological phenomena (e.g., localized Ca^{2+} rises) the intracellular location can be unambiguously identified. In the past years, the imaging technology (traditional and two-photon confocal microscopy, deconvolution of wide-field images, etc.) has significantly improved, thus increasing the need for dyes capable of specifically labeling the various subcellular structures. GFP, the emerging tool in cell biology, to which this volume is devoted, appears ideally suited for this task. In fact, its

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fluorescence depends on an internal chromophore (thus does not require the addition of a cofactor), is not species specific (its simple recombinant expression yields a strong fluorescence signal in cell systems as diverse as bacteria, yeasts, plants, and mammalian cells), and is very resistant to photobleaching. Moreover, its sequence can be modified and appropriate targeting information can be added. In this chapter, we will show that, through suitable chimeras, GFP can be targeted to various intracellular locations (the cytosol, the nucleus, the mitochondria, the endoplasmic reticulum, the Golgi, the subplasmalemmal space), thus providing powerful *in vivo* markers of these compartments.

II. Construction and Expression of the Organelle-Targeted GFP Chimeras

The Cytosol. The starting point for the various GFP chimeras described in this article is the S65T mutant of GFP (Heim *et al.*, 1995). The GFP(S65T) cDNA, which will be also referred to in this chapter as cytGFP, has been modified at the 5′ end of the coding region to include the HA1 epitope tag and an appropriate cloning site (Fig. 1A); when expressed in mammalian cells, the recombinant protein shows mainly a cytosolic distribution with no nuclear exclusion (Fig. 2A). This modified cDNA, encoding a GFP moiety with the HA1

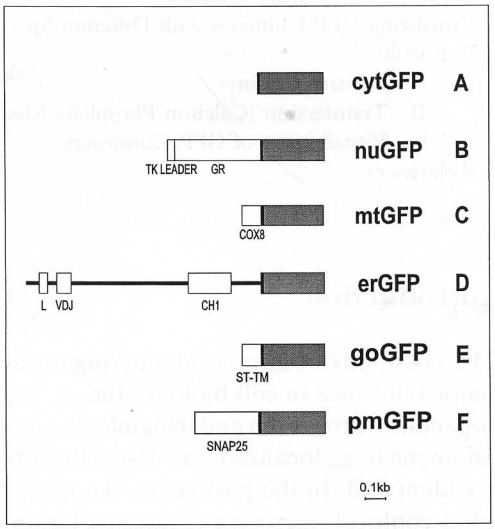


Fig. 1 Schematic representation of the GFP chimeras: cytGFP (A); nuGFP (B); mtGFP (C); erGFP (D) goGFP (E); pmGFP (F). The GFP moiety is indicated in gray; the HA1 epitope is indicated in black.

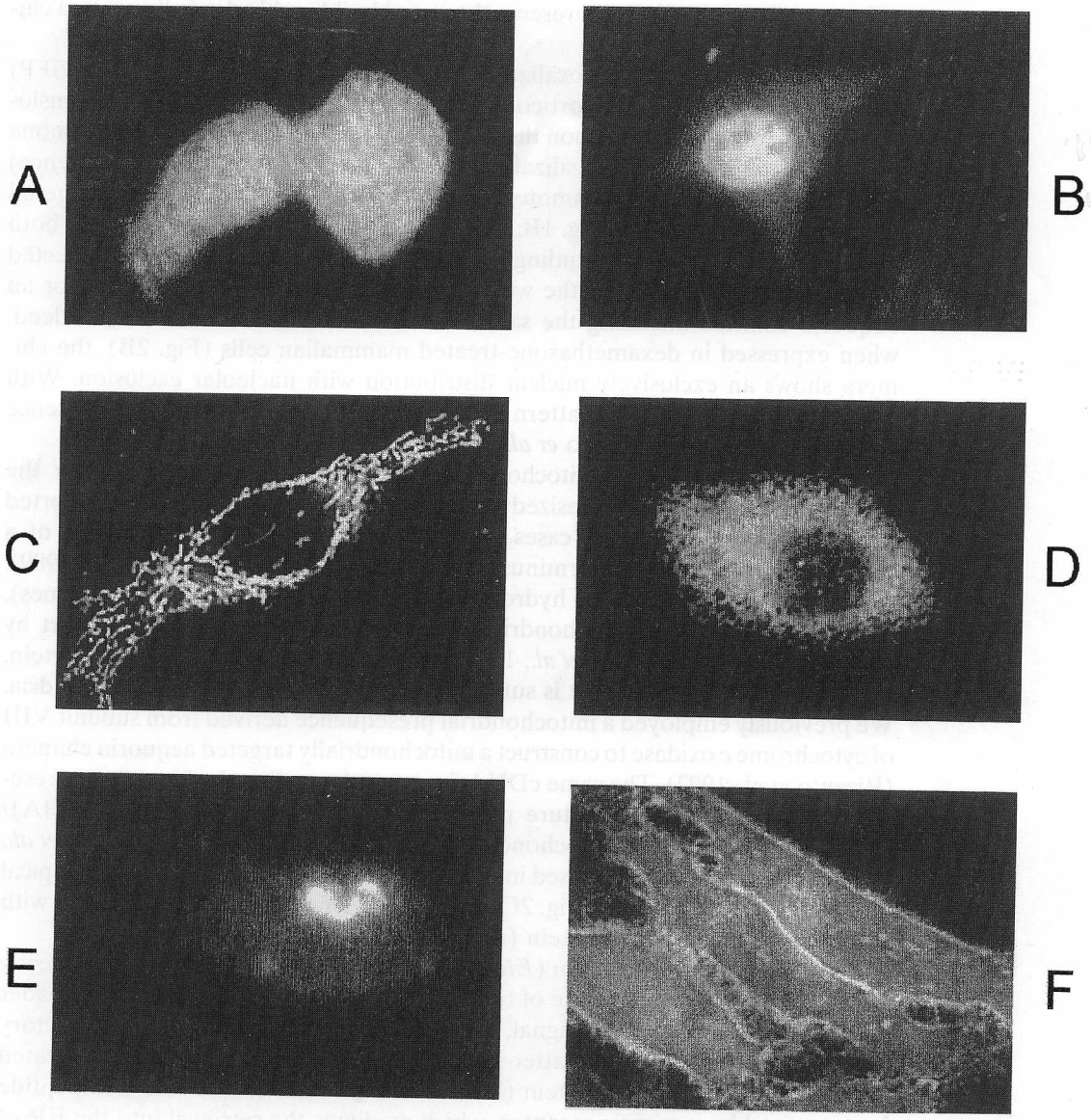


Fig. 2 HeLa cells expressing organelle-targeted GFP: cytGFP (A), nuGFP (B), mtGFP (C), erGFP (D), goGFP (E), pmGFP (F). Cells have been observed 36 h after transfection using the CCD camera. The nuGFP-expressing cell has been treated for 4 h with 10 μM dexamethasone.

epitope at the N terminus represents the initial building block of all targeted chimeras.

The Nucleus. The nuclear localization signal of nuclear-targeted GFP (nuGFP) was obtained from the glucocorticoid receptor (GR), a polypeptide that is translocated to the nucleus when, upon the conformational change induced by hormone binding, a typical nuclear localization signal (aa 497–795 of the GR sequence) is exposed (Picard and Yamamoto, 1987). A schematic map of nuclear-targeted GFP (nuGFP) is shown in Fig. 1B: nuGFP includes aa 407–794 of GR (i.e., both the NLS and the hormone-binding domain) and GFP, and thus can be expected to retain the sorting fate of the wild-type GR, as was previously shown for an aequorin chimera including the same GR domain (Brini *et al.,* 1993). Indeed, when expressed in dexamethasone-treated mammalian cells (Fig. 2B), the chimera shows an exclusively nuclear distribution with nucleolar exclusion. With wild-type GFP, the staining pattern was the same, but the intensity of fluorescence was markedly lower (Rizzuto *et al.,* 1996).

The Mitochondria. All mitochondrial proteins but the 13 encoded by the organellar genome are synthesized on cytoplasmic ribosomes and then imported into the organelle. In most cases, importation depends on the presence of a cleavable signal at the N terminus of a precursor protein (Hartl *et al.,* 1989). This signal (rich in basic and hydroxylated residues, and devoid of acidic ones), usually referred to as mitochondrial presequence, is removed after import by matrix proteases (Hendrick *et al.,* 1989). When added to a heterologous protein, a mitochondrial presequence is sufficient to drive its import into mitochondria. We previously employed a mitochondrial presequence derived from subunit VIII of cytochrome *c* oxidase to construct a mitochondrially targeted aequorin chimera (Rizzuto *et al.,* 1992). The same cDNA fragment (encoding the 25-aa-long presequence and 6 aa of the mature polypeptide) was fused in frame with HA1/GFP(S65T) to construct mitochondrially targeted GFP (mtGFP) (Rizzuto *et al.,* 1995) (Fig. 1C). When expressed in mammalian cells, the chimera shows a typical mitochondrial distribution (Fig. 2C), as is also verified by the colocalization with a mitochondrial resident protein (not shown).

The Endoplasmic Reticulum (*ER*). Resident ER proteins are usually correctly sorted because of the presence of two signals: an N-terminal hydrophobic leader sequence, and a "retention" signal, which prevents their escape into the secretory pathway. In most cases, the latter signal is a defined sequence (KDEL) located at the C terminus of the protein (Munro and Pelham, 1987). This tetrapeptide is recognized by a specific receptor, which mediates the retrieval into the ER of the protein. In other cases, however, the retention signal is not at the C terminus but is located in internal domains of the protein. One of these cases is the immunoglobulin heavy chain, which, in the absence of the light chain, is retained in the ER because of the binding of the CH1 domain to the resident ER protein BiP (Sitia and Meldolesi, 1992). This binding is displaced only by the light chain; in cells that don't express the latter polypeptide, the Ig heavy chain can thus be considered a bona fide ER protein. To include all the targeting information at one end of the protein, we decided to take advantage of this sorting mechanism.

The ER-targeted GFP chimera (erGFP) thus includes a portion of the μ heavy chain (leader, VDJ and CH1 domain) and GFP (Fig. 1D). When expressed in mammalian cells, the strong fluorescent labeling shows the typical reticular pattern of ER (Fig. 2D). As previously shown for a similar aequorin chimera (Montero *et al.,* 1995), this staining overlaps with that of calreticulin, an ER-resident Ca^{2+}-binding protein. In the case of erGFP, a dramatic difference was observed in the fluorescent signal of the chimera including wild-type GFP and the S65T mutant. Immunofluorescence revealed that the recombinant protein was properly sorted and expressed at comparable levels in the two cases. However, although with erGFPwt very few, weakly fluorescent cells could be identified, with erGFP(S65T) a strong labeling was observed in ~50% of the cells. The difference in fluorescence intensity, much more pronounced than with the other organelle-targeted chimeras, may depend, at least in part, on the different rate of chromophore formation (Heim *et al.,* 1995), which may be crucial in the intralumenal environment of the ER.

The Golgi. The Golgi retention of glycosylation enzymes resident in the Golgi has been shown to depend on their single transmembrane domain (TMD), in a process in which the length of the TMD appears to play a key role (Masibay *et al.,* 1993). Sialyltransferase (ST) has been employed as a model system for these studies, and, indeed, it has been shown that the TMD of ST causes the retention of heterologous proteins in the Golgi (Schwientek *et al.,* 1995). We thus constructed a cDNA chimera encoding the TMD of ST fused to GFP (Fig. 1E). When expressed in mammalian cells, the recombinant fluorescent protein appears largely confined to the Golgi (Fig. 2E), although in some cells a weak staining of the ER can also be observed.

The Plasma Membrane. Two different approaches could be employed for targeting GFP to the inner face of the plasma membrane: (*a*) fusing GFP to a cytosolic domain of an integral plasma membrane protein or (*b*) fusing to GFP the signal that recruits cytosolic proteins to this restricted space. Our past experience with aequorin chimeras showed that the latter approach is significantly more efficient. In fact, whereas a glutamate receptor/aequorin chimera was only partly sorted to the plasma membrane (Rizzuto, R., unpublished observations), a fusion protein including aequorin and SNAP25 (a polypeptide that is posttranslationally recruited to the plasma membrane upon palmitoylation of four internal cisteine residues), was fully sorted to the subplasmalemmal region (Marsault *et al.,* 1997). Figure 1F shows the schematic map of the plasma-membrane-targeted GFP (pmGFP). When pmGFP was expressed in mammalian cells, a clear membrane staining could be observed (Fig. 2F), which was also confirmed by the confocal analysis of the transfected cells.

III. Dynamic Monitoring of Organelle Structure with the Targeted GFPs

The GFP chimeras described in this chapter are currently utilized in our lab for monitoring the dynamic changes of organelle structure that occur in living

cells. For this purpose, the cells are seeded onto round coverslips (diameter 24 mm) and transfected with the various GFP constructs, as described in the Section VI, "Protocols." The coverslip is fitted at the base of a thermostatted chamber, which is placed in the microscope stage. Two types of instruments are currently used for detecting GFP fluorescence: (*a*) a wide-field fluorescence imaging system, based on a Zeiss Axiovert inverted microscope and a Princeton Instruments back-illuminated camera, and (*b*) a Nikon RCM 8000 confocal microscope. In both cases, the microscope is equipped with motorized control of the Z stage, thus allowing the acquisition of three-dimensional images. Figure 3 shows the result of a typical experiment carried out (i.e., the dynamic monitoring of ER structure in Cos-7 cells, as obtained by confocal imaging of erGFP expressing cells). It is obvious that by confocal imaging, the dense ER structure of HeLa cells can be finely resolved, and the continuous rearrangement of organelle structure (the three images of the panel are taken 5 min apart) can be clearly appreciated.

IV. Expression in Primary Cultures

Figure 2 showed the expression of the GFP chimeras in the HeLa cell line using standard transfection procedures (see Section VI, "Protocols"). Similar results were obtained with a large variety of cell lines of different embryological origin: CHO (epithelial), L929 (fibroblast), N13 (glial), PC12 (chromaffin), and others (data not shown). Figure 4 (see color plate) shows that this approach can easily be extended to the study of primary cultures, which often require specific culturing procedures.

Figure 4A refers to the expression of the nuGFP chimera in a primary culture of skeletal myotubes. Under appropriate experimental conditions (see Section

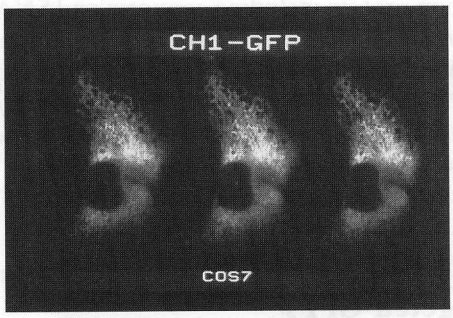
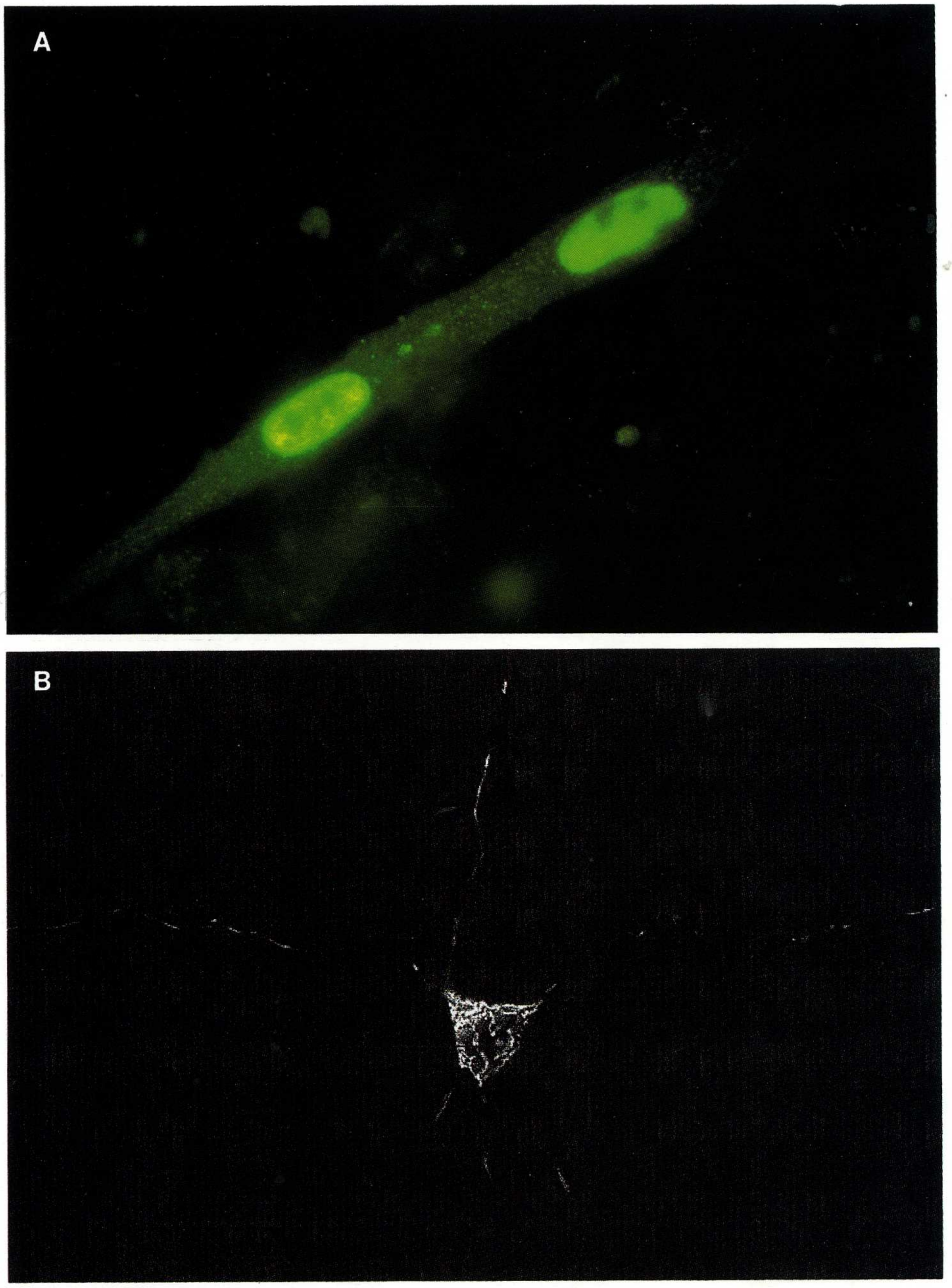
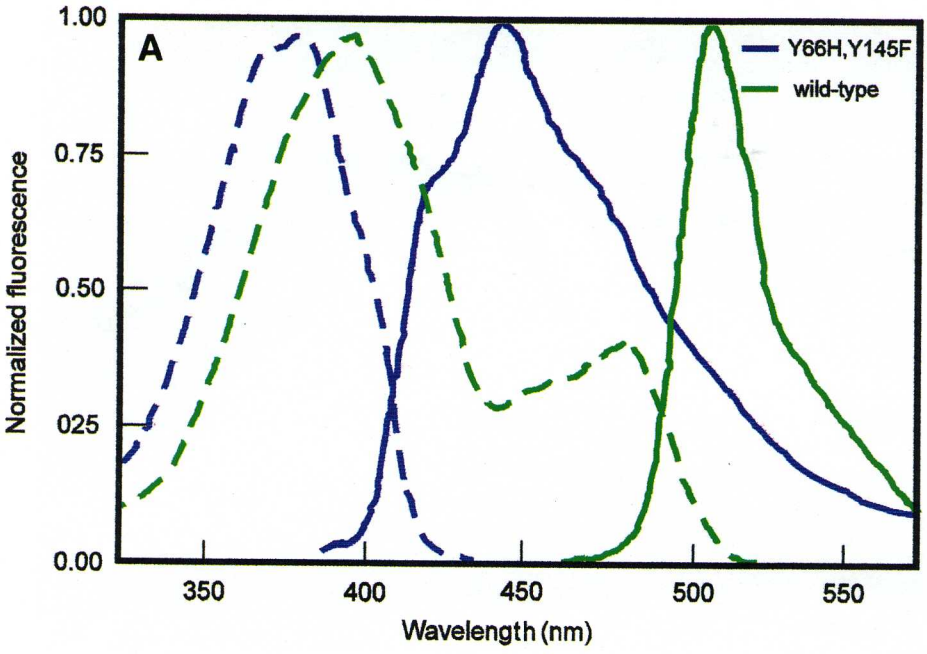
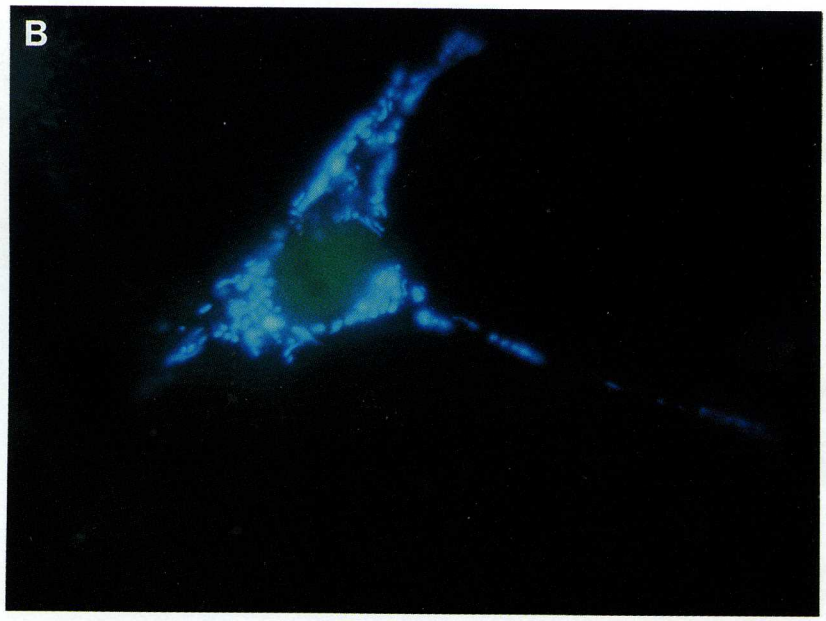


Fig. 3 Dynamic changes of the ER structure in a Cos7 cell in resting conditions: The three images correspond to the same cell expressing erGFP, observed on the confocal microscope at 5-min intervals.



Ch.5, Fig. 4 Expression of organelle-targeted GFP in primary cultures: (A) rat skeletal myotubes transfected with nuGFP. Cells have been treated for 4 h with dexamethasone to induce the translocation of the chimera into the nucleus. (B) Cortical neuron expressing mtGFP.





Ch.5, Fig. 5 (A) Excitation and emission spectra of GFPwt and GFP(Y66H, Y145F), respectively. (B) Cos7 cell cotransfected with nuGFP and mtGFP(Y66H, Y145F) and illuminated with UV light.

VI, "Protocols"), the expression of a recombinant protein can be restricted, in the mixed culture, to the differentiated myotubes. Indeed, the fluorescent live image of a coverslip of cells reveals a strong, specific labeling of the nuclei of transfected myotubes. Figure 4B shows the expression of mtGFP in a primary culture of cortical neurons derived from rat embryos. Cells were transfected at the fifth or sixth day of culture, and the image was taken 36 h after the same transfection procedure employed for HeLa cells. The fluorescent labeling of the mitochondria both in the soma and in the fine processes can be clearly appreciated. Though for brevity no other example is provided, the technique also proved successful with the other chimeras in a variety of other cell lines and primary cultures (e.g., hepatocytes, hepatic ductal cells, etc.). Thus, although a transfection step is necessary, in our experience this does not represent a major limitation, and targeted GFPs may be regarded as a simple and powerful tool for labeling the various organelles of a living cell.

V. Visualizing GFP Chimeras with Different Spectral Properties

Although GFP itself rapidly emerged as an exciting new tool in cell biology, the successful isolation, in the past years, of various useful mutants has further expanded the possible applications. A first group of mutants essentially improves GFP light emission; among those, the S65T mutant, which, as the wild-type protein, can be excited with blue light and emits green light (peak ~510 nm), is in most applications (including the targeted chimeras described earlier) the GFP moiety of choice, thanks to the higher extinction coefficient and faster rate of chromophore formation. A second group of mutants also appears extremely interesting (i.e., those characterized by the possibility of being excited and/or emitting light of different wavelength) because they open the possibility of differentially labeling two or more structures of the same cell. The GFP mutants will be covered in detail by another chapter of this book, so we will not describe them in detail. We will rather provide a simple example of how the combined use of two GFP variants with different spectral properties allows us to unambiguously label two different intracellular organelles, namely the nucleus and the mitochondria. For this purpose, we employed the (Y66H,Y145F) mutant, which, when excited in the UV, emits blue light (peak ~445 nm) and thus can easily be distinguished from the green GFPs (wt or S65T). Figure 5A (see color plate) shows the fluorescence spectra of the two GFP moieties, thus revealing the major shift in the emission (~65 nm at the peak). Figure 5B (see color plate) shows the fluorescence image of a HeLa cell, transiently expressing nuGFPwt and mtGFP (Y66H,Y145F) upon illumination with UV light. The two GFP moieties can clearly be distinguished, thus allowing specific labeling of the two organelles.

VI. Protocols

A. Primary Cultures

1. Cultures of Rat Myotubes (Brini *et al.*, 1997)

Day 1

Remove posterior limb muscle from newborn rats (2–3 days).

Mechanically dissociate cells.

Incubate for 5 min with 0.125% trypsin in phosphate-buffered saline (PBS) and discard the harvest (containing mostly fibroblasts and endothelial cells).

Dissociate muscle cells by three successive 20-min treatments with 0.125% trypsin in PBS and collect the harvests, neutralizing trypsin with fetal calf serum (FCS).

Filter the cell suspension through a double gauze.

Collect cells by centrifugation (10 min at 1200 rpm in a Haereus Minifuge GL).

Resuspend cells in Dulbecco's modified Eagle medium (DMEM) supplemented by 10% FCS and 4.5 g/liter glucose.

Plate in 10-cm Petri dishes at a density of 10^6 cells/dish and incubate for 1 h at 37°C to decrease the number of fibroblasts.

Collect the nonadherent cells and seed at the density of 2×10^6 cells onto 24-mm coverslips coated with 2% gelatin.

Day 3

Change the medium.

Perform transfection as described here.

Day 4

Change the medium with DMEM supplemented with 10% horse serum to increase myoblast fusion.

Day 5

Add to the medium 5 μM 1β-D-arabinofuranosylcytosine to decrease the number of fibroblasts.

Day 6

Change the medium with a DMEM supplemented with 2% horse serum and keep under these conditions.

2. Cultures of Cortical Neurons

Day 1

Remove the uterus from a Wistar timed pregnant female rat (16–17 days of gestation).

Transfer the embryos in a 10-cm Petri dish with cold PBS supplemented with 6 mM glucose and 1% bovine serum albumin (BSA).

Remove the brains from the embryos and put them in the same buffer.

Using a dissection microscope and optical fibers, remove the meninges, extract the cortical regions, and incubate them in a 35-mm Petri dish containing the same cold buffer.

Transfer the cortex in a tube and incubate for 6–7 min with 4–5 ml of PBS supplemented with 0.33 mg/ml papain (prepare it 1 h before use and store at 37°C).

Change the enzyme solution with PBS and dissociate the cells by 5 or 6 passages through a narrowed bore of a fire-polished pasteur pipette.

Centrifuge cells for 5 min at 800 rpm in a cell centrifuge (Haereus Minifuge GL).

Resuspend in modified DMEM (Brewer and Cotman, 1989).

Plate the cells in 24-mm-diameter coverslips pretreated overnight with polylysine at the density of 2.5–3 $\times$ 10^6 cells/coverslip.

Change the medium when living cells are attached (normally after 4–5 h).

Day 2

After 18 h add 5 μM 1β-D-arabinofuranosylcytosine to the medium to block the glial cells grown.

Day 6–7

Perform transfection as described later.

B. Transfection (Calcium Phosphate Method)

Day 1

Precipitate 4–8 μg of plasmid DNA for each coverslip in 70% ethanol, incubate at −80°C for 30 min, centrifuge at 14,000 rpm in a Microcentrifuge Eppendorf, remove the ethanol, and let the pellet dry for 30–40 min.

Resuspend the DNA in 180 μl of TE (10 mM Tris, 1 mM EDTA, pH 8) and then add 20 μl of a 2.5 M $CaCl_2$ solution.

Add this solution dropwise under vortexing to a tube containing 200 μl of 2$\times$ HBS (280 mM NaCl, 50 mM HEPES, 1 mM Na_2HPO_4, pH 7.12 at 25°C).

Incubate for 30 min at room temperature.

Add the DNA precipitate to the coverslip dropwise.

Day 2

Change the medium.

C. Visualization of GFP Constructs

Mount a coverslip with transfected cells, after 36 h of transfection, on a thermostatted chamber containing saline buffer and observe with one of following systems.

1. Fluorescence Microscope

We use a Zeiss Axiovert inverted microscope with a mercury lamp as the light source.

For GFP S65T we use the following filter set:

excitation HQ480/40

dichroic Q480LP

emission HQ510LP

For BFP we use the following filter set:

excitation D390/20

dichroic 425DCLP

emission D460/50

For contemporary visualization of GFPwt and BFP:

excitation D360/40

dichroic 400DCLP

emission E420LP

All the filter sets are provided from Chroma Technology Corporation.

2. Videoimaging

The microscope previously described is equipped with the following devices to form a system for high-speed acquisition and processing of fluorescence images:

- A computer-controlled light shutter.
- A six-position filter wheel.
- A piezoeletric z-axis focus device.
- A back-illuminated 1000 × 800 CCD camera (Princeton Instruments)
- A computer equipped with a software for image acquisition, 2-D and 3-D visualization and analysis (Metamorph software).

3. Confocal Microscopy

We employ the Nikon RCM 8000 confocal microscope based on the Nikon Diaphot 300 inverted microscope, equipped with a 40× water immersion objective (NA = 1.1). The 365-nm band of an argon ion laser is used for excitation of wtGFP and BFP, and the emitted light, separated in two components by a dichroic mirror (475DXCR), is collected by two separate photomultipliers. For visualizing the GFPS65T fluorescence, the 488 band of a helium–neon laser is used and the emitted light (emission filter HQ525/50) is collected by one of the photomultipliers.

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