

Generation of optimized yellow and red fluorescent proteins with distinct subcellular localization

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Fluorescent proteins (FPs) have revolutionized many aspects of cell biology and have become indispensable research tools. Today's increasingly complex experiments aiming to understand biological systems strongly depend on the availability of combinations of multiple FPs, which allow their distinguishable simultaneous detection in the same cell or tissue. Recently, the VENUS and DsRed.T4 FPs were described as the latest generation of yellow and red FPs. To increase the combinatorial possibilities when using these optimized FPs, we have generated and successfully tested seven new forms of VENUS and DsRed.T4 proteins with distinct subcellular localization. To facilitate their use as markers in biological experiments, bicistronic expression constructs, which have been optimized for robust expression in almost all mammalian developmental stages and cell types, were produced for the new FPs. In addition, several plasmids were created, which contain all necessary elements for inserting the reading frames of these FPs into specific gene loci in knock-in experiments without disrupting the reading frame of the endogenous gene.

INTRODUCTION

The availability of fluorescent proteins (FPs) has revolutionized many aspects of cell biology. The possibility to noninvasively detect an expressed FP as a marker in living cells without further staining has made them indispensable tools in many areas of experimental biology (1). For example, linking the expression of an FP to the expression of an exogenous gene in a bicistronic construct using an internal ribosomal entry site (IRES) allows easy detection and isolation of cells expressing this construct after transfection. FPs have also successfully been used to mark cells of specific lineages in vitro and in vivo by inserting open reading frames of FPs into the genomic loci of genes specifically expressed in cells of a certain lineage (2,3). However, to understand the regulation of biological systems, it is essential to analyze the effects of more than only one gene. Accordingly, multiple distinguishable FPs are necessary to independently detect the expression of, for example, different ectopically or endogenously expressed genes (4,5). The use of FPs with distinct spectral

properties allows simultaneous and distinguishable detection in the same cell (reviewed in Reference 6), both for microscopic analysis and fluorescence-activated cell sorting (FACS). In addition, the number of FPs for simultaneous use in one experiment can be increased further by using FPs with distinct subcellular localizations when using microscopic analysis.

In most biological experiments, the properties of the used FPs will be the main limiting factor in experimental design, making it essential to choose the best available FP combinations. Although being the most widely used FP, enhanced green fluorescent protein (EGFP) has several disadvantageous spectral properties: (i) its emission spectrum strongly overlaps with those of enhanced cyan fluorescence protein (ECFP) and enhanced yellow fluorescent protein (EYFP), preventing simultaneous use with either of them unless highly specialized technology (e.g., spectral linear unmixing) is used for detection; and (ii) compared to EYFP and DsRed, the shorter excitation and emission wavelengths of EGFP leads to higher autofluorescent background of biological samples and to lower tissue

penetration. Therefore, their spectral properties would make EYFP and DsRed better markers for use in multi-color experiments than EGFP.

Recently, several problems of previous YFP and DsRed forms have been overcome by the development of improved versions of EYFP (VENUS) (7,8) and DsRed (DsRed.T4) (9). The Venus protein differs from EGFP and EYFP by 8 and 5 amino acid exchanges, respectively. It has the same spectral properties as EYFP, but it has a 30-fold higher relative fluorescence, it matures faster and is less sensitive to pH and chloride ion concentrations (7). As a derivative of the extensively tested EGFP, the brighter VENUS is most likely to be better suited as a marker in living cells of many lineages. Accordingly, VENUS has successfully been used for marking specific cell lineages in transgenic mice (M. Osawa, personal communication) or cell lineages derived from ES cells by insertion into lineage-specific gene loci (I. Samokhvalov, personal communication) without any apparent toxic effects. DsRed.T4 [almost identical to DsRed.T1 (9), which is now sold as DsRedExpress by BD Biosciences Clontech (Palo Alto, CA, USA)], matures much faster than earlier DsRed versions. In addition, while earlier DsRed proteins also formed an intermediate emitting green light, this intermediate is not detectable with DsRed.T4. Upon expression in cells, these properties lead to a stronger and cleaner red fluorescent signal of DsRed.T4 than of earlier DsRed proteins (9). Moreover, it has a lower tendency to form aggregates, which was the major reason for the toxicity of earlier DsRed versions (9). In line with this, high stable expression of DsRed.T4 cells has no toxic effect in undifferentiated ES cells and during their differentiation in vitro (T. Era, personal communication).

However, when using the wild-type forms of VENUS and DsRed.T4, experiments are usually limited to the use of two or three (together with ECFP) FP markers in living cells. In many current and future research projects analyzing more complex living biological systems under physiological conditions, more markers are needed. In addition, it must be noted that while ECFP can

be used as a third marker together with VENUS and DsRed, its short excitation and emission wavelengths lead to high autofluorescent background of most biological samples, making its use extremely difficult or impossible in many experimental settings. Other possibilities to combine better markers like VENUS and DsRed.T4 in one experiment would therefore be advantageous. To this end, we have generated a panel of plasmids encoding new VENUS and DsRed.T4 fusion proteins that are targeted to distinct subcellular compartments. This now makes it possible to simultaneously mark the expression of up to five (or six when using confocal microscopy) gene loci with these state-of-the-art FPs.

MATERIALS AND METHODS

Transfections

Semiconfluent human 293T cells and murine OP9 cells were transfected with expression constructs for the different new FPs. Two micrograms of each DNA were transfected per 3-cm plate of cells using the Mirus TransIT[®]-LT1 lipofection kit according to manufacturer's instructions (Takara, Otsu, Japan). For pentuple labeling, OP9 cells in 3-cm plates were transfected with pCAG-IRES-FP expression vec-

tors for 1.5 μ g VENUSnucmem, 2 μ g mitoVENUS, 4 μ g golgiVENUS, 1 μ g mitoDsRed.T4, and 3 μ g DsRed.T4nuc expression vectors.

Image Acquisition and Processing

Epifluorescent pictures were taken 24 h after transfection with an Axiovert 200 microscope (original magnification 63-fold) (Carl Zeiss Japan, Tokyo, Japan) equipped with halogen and mercury lamps, tetramethyl rhodamine isothiocyanate (TRITC) filter set 15 (Carl Zeiss), Chroma YFP filter sets (Rockingham, VT, USA), the AxioCam HRc camera (monochrome mode), and Axiovision software (both from Carl Zeiss). The experiment was repeated with identical results and typical pictures are shown, respectively.

Fluorescence-Activated Cell Sorting Analysis

293T cells were suspended in phosphate-buffered saline (PBS) 24 h after transfection and analyzed for fluorescence intensity using a FACSCalibur[™] machine (BD Biosciences, San Jose, CA USA) with standard FL-1 and FL-2 filter settings. CellQuest[™] software (BD Biosciences) was used for data acquisition, WinMDI software for data analysis. The experiment was repeated with identical results.

RESULTS AND DISCUSSION

To create new VENUS and DsRed.T4 fluorescent proteins with specific subcellular localization, we made use of targeting signal sequences that had successfully been used for the respective subcellular forms of GFP earlier (<http://www.bdbiosciences.com/clontech> and references below). Nuclear forms (VENUSnuc and DsRed.T4nuc) were created by fusing a nuclear localization signal of simian virus 40 (SV40) (PK-KKKRV) to the C terminus of the FPs. Mitochondrial forms (mitoVENUS and mitoDsRed.T4) were created by an N-terminal fusion of amino acids 2–29 of the human cytochrome C oxidase subunit VIII [reverse transcription PCR (RT-PCR) from 293T cDNA] (10). The golgi forms (golgiVENUS and golgiDsRed.T4) were produced by fusing the N-terminal 81 amino acids of human β -1,4-galactosyltransferase (RT-PCR from 293T cDNA) to the N terminus of the FPs (11). Chromatin-localized VENUS (H2BVENUS) was created by N-terminal fusion of human histone 2B (12) and VENUS localized to the nuclear membrane (VENUS-nucmem) by a C-terminal fusion of amino acids 2–67 of human Importin α -1 (RT-PCR from 293T cDNA) (Reference 13 and K. McNagny and T. Graf, personal communication). To create plasmids for bicistronic expression with other genes, reading frames of the original and the new forms of VENUS and DsRed.T4 were cloned behind an encephalomyocarditis virus IRES sequence (IRES2 from plasmid pIRES2-EGFP; BD Biosciences Clontech). Care was taken to place the start codons of the FP reading frames onto the same optimized start codon of the IRES as for the ATG of EGFP in the original plasmid. For robust expression, the IRES-FP combinations were then cloned into pCAG vector backbones, which yield high expression in most cell types in vitro and in vivo (14). Importantly, and in contrast to most other promoter/enhancer combinations, expression from these backbones is also maintained during differentiation of ES cells into mature adult type cells in vitro and in vivo (15,16). H2BVENUS was cloned into the pCAG backbone

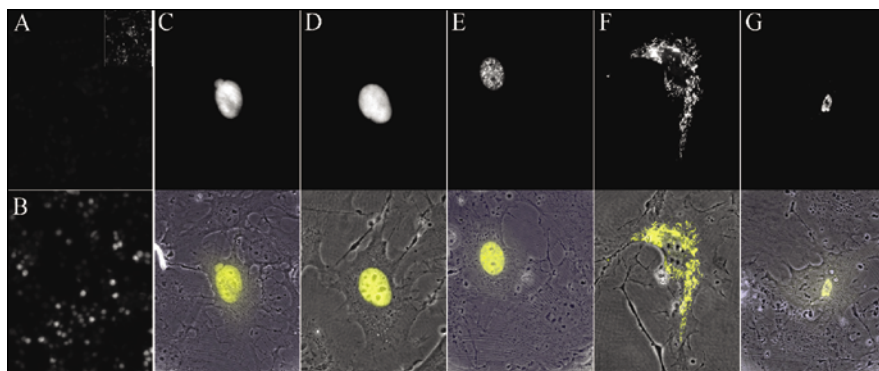


Figure 1. New VENUS fusion proteins with specific subcellular localization. (A and B) Subcellular VENUS forms are brighter than the respective enhanced yellow fluorescent protein (EYFP) forms. 293T cells transfected with pCAG-nucVENUS and pCAG-nucYFP, respectively. Identical conditions were used for all steps from DNA preparation, transfection, fluorescent protein (FP) detection, to final image processing. The insert in (A) shows the same image with increased image enhancement. (C–G) New subcellular VENUS forms in OP9 cells. Upper panels show original monochrome fluorescence pictures after image enhancement. Lower panels show false (yellow) color overlay images with phase contrast pictures of the same cells. All proteins showed the same subcellular distribution after transient transfection in 293T cells (panel B and data not shown). (C) VENUSnuc, (D) VENUSnucmem, (E) H2BVENUS, (F) mitoVENUS, and (G) golgiVENUS.

without an IRES sequence. For easier cloning of an additional gene into these plasmids, a multiple cloning site (5'-*KpnI/NotI*-3' from plasmid pBlueScript® IISK+; Stratagene, La Jolla, CA, USA) was introduced 5' of the IRES site, with some of its enzyme sites being unique. All relevant parts of the resulting plasmids were sequenced for verification, and sequence files are available upon request.

To functionally test the created fusion proteins and expression plasmids, they were transiently transfected into human 293T cells and the murine OP9 stromal cell line (17). All proteins were efficiently expressed from the used promoter-intron-IRES-poly(A) signal combination (Figures 1 and 2). As expected, the subcellular forms of the VENUS protein were much brighter than the respective EYFP forms after expression in mammalian cells (Figure 1, A and B, and data not shown). As shown in Figure 2, VENUS and DsRed.T4 can clearly be distinguished in microscopic and FACS analyses using standard filter settings (Figure 2).

Of all the tested forms, only golgiDsRed.T4 did not show the expected subcellular distribution. Although red fluorescence was detectable in the cells, it had a clumpy distribution, spread all over the cell, and was not focused in the Golgi apparatus (data not shown). It was, therefore, not used for further experiments. The tetrameric nature of DsRed proteins could be a possible explanation for the clumpy and misdirected subcellular localization of the fusion protein. Depending on the proliferative status of the cultures, leaking of the VENUSnuc and DsRed.T4nuc forms into the cytoplasm was detected in some cells. This could only be observed in proliferating cultures and is likely to happen during the breakdown of the nuclear membrane during mitosis. In contrast, VENUSnucmem was always exclusively localized to the nuclear membrane in all cells and will therefore usually be the better nuclear marker in proliferating cells. Quantification of fluorescence intensity of the transfected cultures by FACS analysis revealed that transfection with VENUSnucmem yields slightly higher fluorescence than with mitoVENUS,

VENUSnuc, and golgiVENUS, in this order (data not shown). Similarly, mitoDsRed.T4 yields slightly higher fluorescence than DsRed.T4nuc (data not shown). However, it is important to point out that even the relatively darker

golgiVENUS and DsRed.T4nuc still exhibit very bright fluorescence compared to other fluorescent proteins and can easily be detected (Figure 2 and data not shown).

The major advantage to using FPs

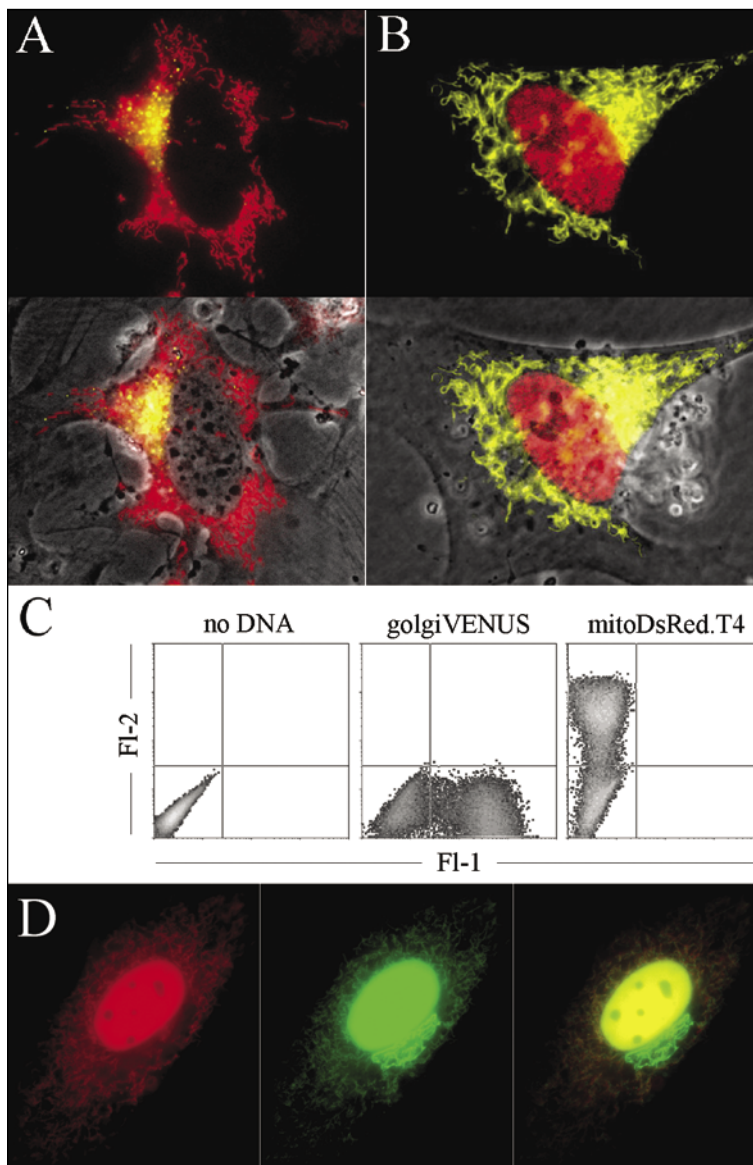


Figure 2. New DsRed.T4 and VENUS proteins with specific subcellular localization allow multiple simultaneous labeling. (A and B) New subcellular DsRed.T4 forms in OP9 cells. Upper panels show false color overlay images [yellow and red for images taken with the yellow fluorescent protein (YFP) and tetramethyl rhodamine isothiocyanate (TRITC) filter sets, respectively] of the fluorescent images after image enhancement. Lower panels show false color overlay images with the phase contrast pictures of the same cells. (A) mitoDsRed.T4 and golgiVENUS. (B) DsRed.T4nuc and mitoVENUS. (C) VENUS and DsRed.T4 can be distinguished in standard fluorescence-activated cell sorting (FACS) analyses. 293T cells were transfected with expression constructs for VENUS or DsRed.T4 forms. The FI-1 and FI-2 channels of a FACSCalibur machine with standard filter settings were used for detection. (D) Pentuple staining of an OP9 cell transfected with VENUSnucmem, mitoVENUS, golgiVENUS, mitoDsRed.T4, and DsRed.T4nuc expression vectors. Left and middle panel: red and green false color images taken with the TRITC and YFP filter sets, respectively (overlaid in the right panel).

Table 1. Available Plasmids for Expression of New Fluorescent Proteins

FP	pUC19-IRES- FP-lox-neo-lox	pCAG-FP	pCAG-IRES-FP
VENUS			
Nuclear	+		+
Nuclear membrane	+		+
Histone		+	
Mitochondrial		+	+
Golgi	+		+
DsRed.T4			
Nuclear			+
Mitochondrial	+		+

FP, fluorescent protein; IRES, internal ribosomal entry site.

with distinguishable spectra and subcellular localization lies in the ability to add multiple additional markers in future experiments. To avoid limitations in possible future (not yet anticipated) experiments due to the use of a

restrictive marker like EGFP, experiments should be started with a marker like one of the VENUS forms described here, which allow more combinatorial possibilities. While being an important consideration for in vitro experiments with cell lines, this is most critical when creating transgenic animals expressing specific FPs. One main advantage of creating such animals—the possibility to combine their properties (marked with an FP) with those of other animals by mating—becomes obsolete when the same marker like EGFP was used in all animals. The pCAG plasmid, which we chose as the expression construct for all the new

FPs described here, has been shown to yield robust FP expression in virtually all cells of transgenic animals (14). To also facilitate the use of the new subcellular forms of VENUS and DsRed.T4 for the generation of knock-in animals, we constructed several new plasmids. A pGK promoter-driven neomycin resistance gene, flanked by loxP sites for later deletion, was cloned immediately 3' of IRES2-FP combinations for VENUS-nuc, VENUSnucmem, golgiVENUS, and mitoDsRed.T4. All relevant parts were sequenced for verification, and sequence files are available upon request. The use of an IRES for knock-in experiments allows the expression of a gene locus to be detected without the disruption of the endogenous reading frame by the inserted FP reading frame and has successfully been applied in various cell types including olfactory neuronal cells (18) and hematopoietic cells (N. Uyttersprot, personal communication). This is crucial for marking the expression of one or both alleles of genes, which have to be functionally present in a homo- or heterozygous state for normal development of animals or the cell system of interest. In addition, marking of both alleles of a gene will be essential when using a gene locus that is expressed in a monoallelic manner.

The array of new expression plasmids for fluorescent proteins with subcellular localization described here (Table 1) will provide valuable tools for applications in many areas of experimental biology and will be available upon request.

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Annealing control primer system for identification of differentially expressed genes on agarose gels

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We developed GeneFishing technology, an improved method for the identification of differentially expressed genes (DEGs) using our novel annealing control primer (ACP) system. Because of high annealing specificity during PCR using the ACP system, the application of the ACP to DEG discovery generates reproducible, authentic, and long (100 bp to 2 kb) PCR products that are detectable on agarose gels. To demonstrate this method for gene expression profiling, GeneFishing technology was used to detect genes that are differentially expressed during development using total RNAs isolated from mouse conceptus tissues at 4.5–18.5 days of gestation. Ten DEGs (DEG1–10) were isolated and confirmed by Northern blot hybridization. The sequence analysis of these DEGs showed that DEG6 and DEG10 are unknown genes.

INTRODUCTION

Techniques designed to identify differentially expressed genes (DEGs) in cells under various physiological stages or experimental conditions (e.g., during developmental or neoplastic differentiation or pharmacological treatment) have become pivotal in modern biology. The difficulty of identifying (or "fishing out") a gene responsible for a specialized function during a certain biological stage often arises be-

cause the gene is expressed at low levels, whereas the bulk of mRNA within a cell are highly abundant transcripts (1). To screen for DEG transcripts in low concentrations, PCR amplification is required. One screening method, differential display, requires PCR using short arbitrary primers and is described by Liang and Pardee (2). Although this method is simple, rapid, and only requires small amounts of total RNA, many investigators have experienced significantly high false-positive rates