Expression of fluorescently tagged connexins: a novel approach to rescue function of oligomeric DsRed-tagged proteins[1]

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Abstract A novel, brilliantly red fluorescent protein, DsRed has become available recently opening up a wide variety of experimental opportunities for double labeling and fluorescence resonance electron transfer experiments in combination with green fluorescent protein (GFP). Unlike in the case of GFP, proteins tagged with DsRed were often found to aggregate within the cell. Here we report a simple method that allows rescuing the function of an oligomeric protein tagged with DsRed. We demonstrate the feasibility of this approach on the subunit proteins of an oligomeric membrane channel, gap junction connexins. Additionally, DsRed fluorescence was easily detected 12–16 h post transfection, much earlier than previously reported, and could readily be differentiated from co-expressed GFP. Thus, this approach can eliminate the major drawbacks of this highly attractive autofluorescent protein.

Key words: Green fluorescent protein; Red fluorescent protein; Gap junction; Oligomeric membrane protein; Aggregation of protein; Protein chimera

1. Introduction

The past years have seen a revival of fluorescence microscopy, in particular due to the recent availability of the intrinsically fluorescent tracer protein green fluorescent protein (GFP), isolated and cloned from the northwest Pacific bioluminescent cold-water jellyfish *Aequorea victoria* [1,2]. The inert nature of the protein and the many potential uses of GFP, including the possibility to observe the behavior of proteins in living cells was quickly recognized, and triggered the development of novel GFP variants, with improved characteristics, as well as a frantic search for other autofluorescent proteins that fluoresce at different wavelengths than GFP.

Recently, a new red fluorescent protein (RFP, drFP583) was isolated from the Indo–Pacific anthozoa, *Discosoma* spec. that fluoresces brilliantly red ([3], reviewed in [4]). The cDNA sequence of this protein was optimized for mammalian expression and is commercially available as DsRed (Clontech Laboratories, Palo Alto, CA, USA). DsRed attracted tremendous interest as a complementary expression partner to GFP that would easily allow simultaneous multicolor imaging of at least two different proteins in living cells, as well as fluorescence resonance electron transfer (FRET) studies, since its excitation and emission wavelengths are located far away from GFP (enhanced GFP ex./em. max. 488/509 nm; DsRed ex./em. max. 558/583 nm). So far, these approaches were limited to the expression of the cyan- (CFP) and yellow- (YFP) shifted color variants of GFP, a relatively challenging venture due to the similar excitation and emission wavelengths of these variants (CFP ex./em. max. 433/475 nm; YFP ex./em. max. 513/527 nm), rapid bleaching of CFP, and the requirement of an excitation wavelength not emitted by conventional krypton/argon ion lasers widely used in confocal microscope systems [5,6]. Color-shifted variants of DsRed [7,8], and other newly developed properties, such as a slow color change over time [9] promises to make this autofluorescent protein even more valuable.

However, in many cell biological studies, DsRed-tagged proteins were found not to behave as inert as their homologs tagged with GFP, and abnormal localization of the DsRed fusion proteins, and intracellular located aggregates were often observed (e.g. DsRed-eCOPI, J. Lippincott-Schwartz, NIH, Bethesda, MD, USA; GalNAc-T2-DsRed, P. Keller and K. Simons, MPI, Dresden, Germany; histone 2B-DsRed and lamin B receptor-DsRed, J. Ellenberg, EMBL, Heidelberg, Germany; myosinVI-DsRed, T. Hasson, UCSD, USA; DsRed-γ-actin, A. Matus, FMI, Basel, Switzerland; personal communication). Hydrodynamic analysis [7,8], and crystallographic data of the DsRed protein [10,11] revealed that DsRed has a strong oligomerization tendency that results in a homomeric tetramer in vitro, as well as in living cells [7]. This tetramerization tendency is believed to be responsible for the aberrant localization and formation of aggregates of the DsRed-tagged proteins.

2. Materials and methods

2.1. cDNA constructs

Connexin (Cx) cDNAs missing a stop-codon were cloned into the *Eco*RI and *Bam*HI cloning sites of the expression vectors pEGFPN1, and pDsRed1-N1 (Clontech Laboratories, Palo Alto, CA, USA) as described [12]. Fusion proteins consisted of the Cx sequence, a seven amino acid residue linker-region and the enhanced GFP, or DsRed sequences, respectively. Wild-type Cx43 was derived by cloning the Cx43 encoding cDNA including its authentic stop-codon into the pEGFPN1 vector.

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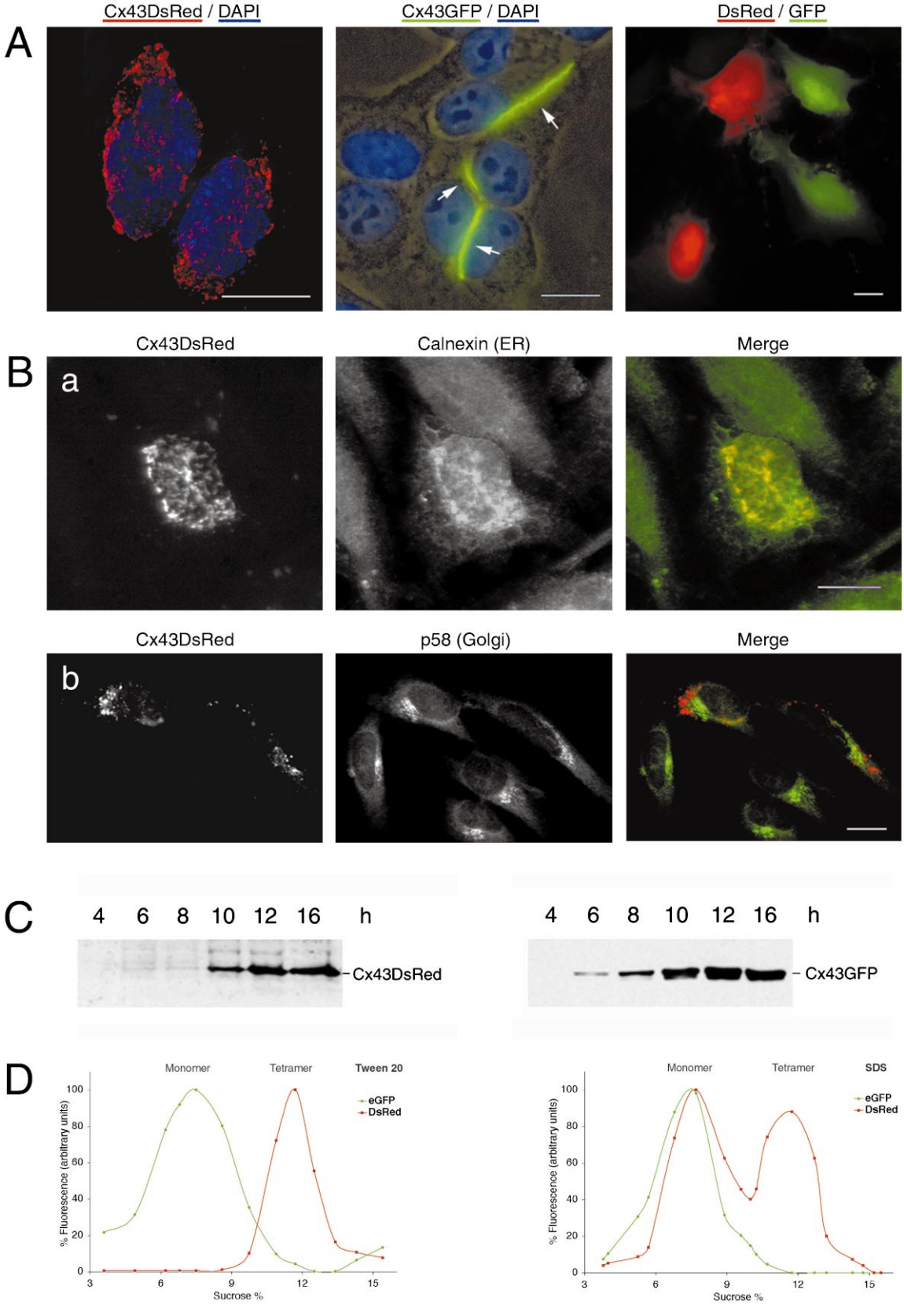
2.2. Cell culture and transient transfections

HeLa (ATCC CCL 2), COS7 (ATTC CRL 1650), and T51B cells [16] were cultured under standard conditions as described [12] and transfected with Superfect® transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

2.3. Fluorescence labeling and microscopy

Cells were grown on poly-L-lysine- (Sigma, St Louis, MO, USA) coated coverglasses, fixed, and immunostained (where appropriate) as described [12]. Polyclonal antibodies directed against calnexin (Stress-Gen, Victoria, BC, Canada) (ER marker), and monoclonal anti-Golgi



p58 protein (Sigma) were used in 1:100 dilution. Nuclei were stained with DAPI before mounting. DsRed, GFP, and immunostained preparations were imaged using a Zeiss Axiovert 35M inverted microscope equipped with a 63×Plan-Apochromat, NA 1.4 oil immersion lens, an HBO 200 W mercury arc lamp for epifluorescence, standard Texas red, FITC, and DAPI filter sets (Chroma Technology Corp., Brattleboro, VT, USA), and a cooled charge-coupled device camera (CCD; Orca-100; Hamamatsu Inc., Bridgewater, NJ, USA). Grayscale digital images were collected separately using Openlab software (Version 2.5.2, Improvision Inc., Lexington, MA, USA). Images were converted into Photoshop 5.02 (Adobe Systems Inc., San Jose, CA, USA) and used as such, or merged in pseudocolors. Images presented in Figs. 1Aa and 2A were acquired and processed using a DeltaVision Model 283 deconvolution microscope system (Applied Precision Inc., Issaquah, WA, USA) as described previously [12].

2.4. Immunoblot analysis

Total cell homogenates of transfected cells were obtained at the times indicated and separated on 10% SDS protein gels, transferred onto nitrocellulose membranes, and blocked with 5% dry milk. Polyclonal anti-GFP and anti-DsRed antibodies (Clontech Laboratories) were used in 1:1000 dilution. Horseradish peroxidase coupled secondary goat anti-rabbit (Bio-Rad, Hercules, CA, USA) was used at 1:15 000 dilution. Bound antibodies were detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

2.5. Hydrodynamic analysis

HeLa cells expressing either DsRed, or GFP were lysed on ice in 0.1% Triton X-100. 100 000×*g* supernatants were incubated for 5 h in 0.1% Tween 20, or 0.1% SDS, respectively, and loaded on separate, linear 5–15% sucrose gradients containing 150 mM NaCl, 50 mM Tris, pH 7.6, and 0.1% Tween 20, or 0.1% SDS. Gradients were centrifuged for 16 h at 16°C at 38 000 rpm in an SW55TI rotor (Beckman Instruments Inc., Palo Alto, CA, USA) as described [13]. 0.4 ml fractions were collected from the bottom, sucrose concentrations determined, and fluorescence emission spectra of each fraction were obtained using a Hitachi F-2000 fluorescence spectrophotometer as described [14]. Excitation wavelength for DsRed was 558 nm, and for enhanced GFP 475 nm. Peaks of GFP and DsRed monomer fluorescence were generally detected at 7.5–8.0% sucrose. A second DsRed emission peak, most likely corresponding to the DsRed tetramer was obtained at 11.4–11.8% sucrose.

3. Results and discussion

3.1. Expression of DsRed-tagged Connexins

When we expressed Cxs, polytopic transmembrane proteins that oligomerize into gap junction channels, tagged with DsRed on their C-termini, aberrant perinuclear located aggregates were visible within 12 h post transfection that quantitatively affected all tagged Cxs. No gap junction channel clusters were assembled in the adjacent plasma membranes of transfected cells, even after prolonged post translation periods (40 h) (Fig. 1Aa). DsRed fusion protein aggregates were formed with all Cx isotypes tested (Cx43, Cx32, Cx26, data shown for Cx43 only), and were not cell type specific (see Section 3.4). On the other hand, Cxs similarly tagged with GFP were unaffected and trafficked and assembled efficiently into functional gap junction channels [12,15,16] (Fig. 1Ab). DsRed and GFP expressed alone were soluble and distributed throughout the entire cytoplasm of expressing cells (Fig. 1Ac). Previous studies have shown that untagged DsRed tetramers are soluble in vitro and in vivo [7,10,11], however, aggregates have been seen in aging *Escherichia coli* cultures expressing DsRed protein [17].

Partial immuno-colocalization with antibodies directed against calnexin, a chaperone-protein localized in the lumen of the endoplasmic reticulum (ER) indicated that DsRed-tagged Cxs aggregated following their translation within this subcellular compartment (Fig. 1Ba). Furthermore, a distinctive change of ER appearance was observed in Cx-DsRed transfected cells, which appears to result from a collapsing of ER membranes in perinuclear regions. No colocalization with downstream transport compartments, such as Golgi membranes, was found (Fig. 1Bb). However, colocalization with additional subcellular components, present within the perinuclear region can not be excluded. Previous studies have shown that Cxs are integrated co-translationally into ER membranes, oligomerize into hexameric hemichannels (termed connexons), and are transported through the Golgi apparatus to reach their functional site in the plasma membrane. There, connexons of neighboring cells dock head-on to form functional transmembrane gap junction channels, which provide direct cell-to-cell communication. Finally, channels arrange into large clusters, termed gap junction plaques (reviewed in [18]).

Western blot analysis of DsRed and GFP-tagged Cxs using DsRed and GFP specific antibodies revealed protein bands that corresponded in size to the expected fusion proteins (Fig. 1C), indicating that the aggregation of DsRed-tagged Cxs is not due to the production of truncated, or otherwise aberrant fusion proteins.

Hydrodynamic analyses of GFP and DsRed isolated with non-ionic detergent (Tween 20) from transfected HeLa tissue culture cells resulted in two peaks, at 7.5 and 11.7% sucrose, probably corresponding to GFP monomer, and DsRed tetramer, respectively (Fig. 1D, left panel). Treatment of the cell lysates with 0.1% SDS for 5 h prior to hydrodynamic analysis resulted in a partial transfer of the tetrameric into monomeric DsRed that co-migrated with monomeric GFP (Fig. 1D, right panel). This result indicates that aggregation of DsRed-tagged Cxs in the ER membranes is also not due to a larger hydrodynamic dimension of the DsRed over the GFP

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Fig. 1. Characterization of DsRed-tagged Cx43 transiently expressed in HeLa tissue culture cells. A: Expression of Cx43DsRed results in an aberrant, quantitative aggregation of the fusion protein in a perinuclear region (a), while Cx43 identically tagged with GFP is unaffected and traffics and assembles efficiently into gap junction plaques (arrows) in the plasma membranes (b). DsRed and GFP expressed alone are soluble and fill the entire cell bodies (c). Images were taken 16 h post-transfection. B: Localization of Cx43DsRed to ER membranes. a: ER was visualized with antibodies directed against calnexin (green). Merge of Cx43DsRed and calnexin fluorescence indicates partial colocalization (yellow). Note the distinctively changed appearance of the ER in Cx43DsRed expressing cells. b: Golgi cisternae were stained with antibodies directed against p58 protein. No colocalization with this downstream transport compartment was detected. Scale bars = 10 μm. C: Immunoblot analysis of DsRed, and GFP-tagged Cx43. Transfected cells were harvested at the indicated time points post-transfection and Cx43DsRed and Cx43GFP fusion proteins were analyzed by Western blot analysis. D: Hydrodynamic analysis of DsRed, and GFP. DsRed and GFP were isolated from transfected HeLa tissue culture cells, incubated in Tween 20, or SDS, respectively, and their relative density was determined by velocity centrifugation. Percent fluorescence at 509 nm (GFP), and 583 nm (DsRed) were blotted against sucrose concentrations. Monomeric GFP, and tetrameric DsRed were obtained in Tween 20. Tetrameric DsRed was partially disassembled into monomers after treatment with SDS.

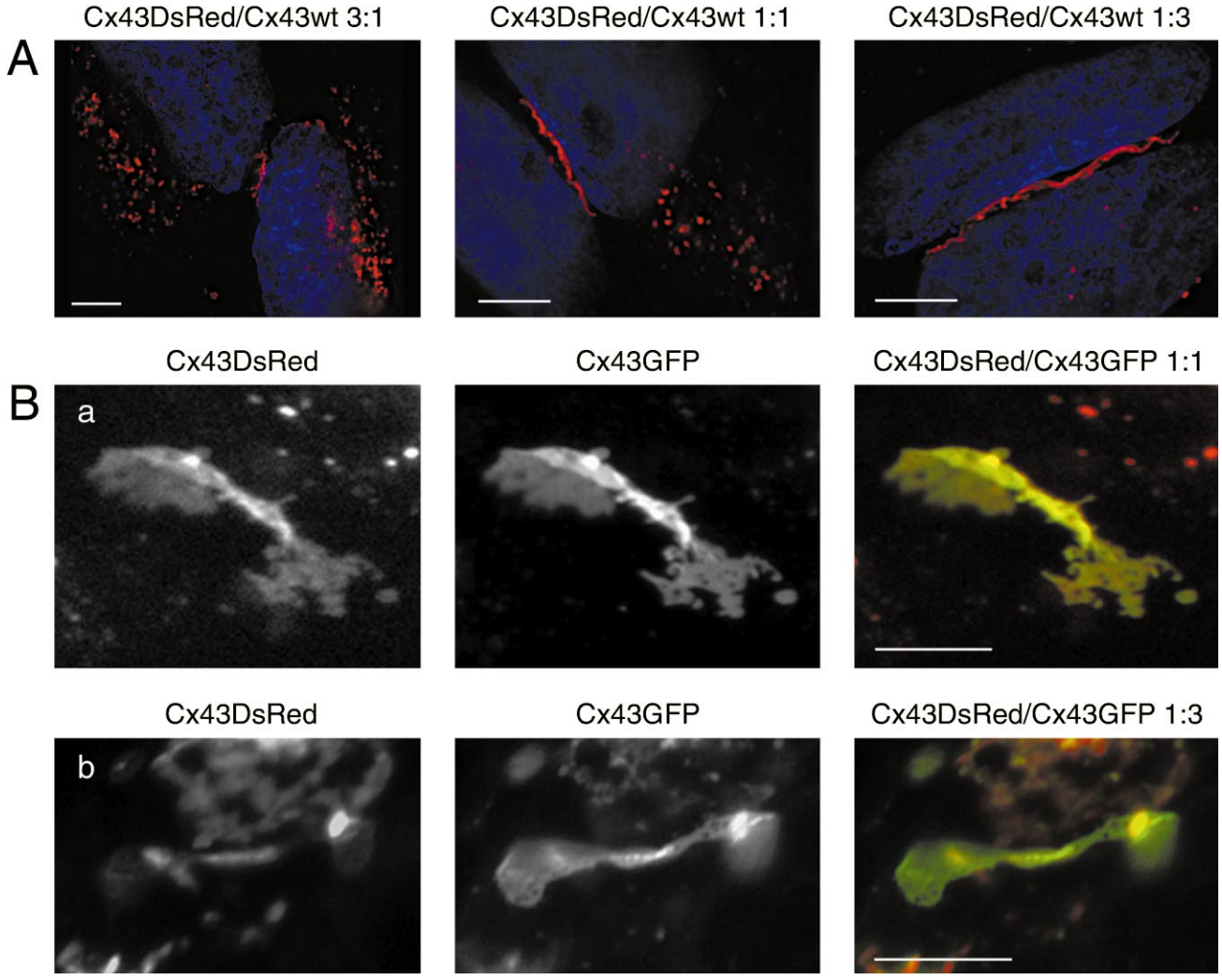

Fig. 2. Rescuing DsRed-tagged Cx function. DsRed-tagged Cxs assemble into gap junction channel plaques at the plasma membranes when co-transfected with wild-type, or GFP-tagged Cxs. A: cDNAs encoding DsRed-tagged and wild-type Cx43 were mixed in the indicated ratios prior to transfection. The size and number of assembled gap junction channel plaques increased with higher amounts of Cx43 wild-type cDNA, and the amount of intracellular aggregates was reduced. B: Gap junction channel plaques were also assembled from co-expressed DsRed and GFP-tagged Cxs. a: cDNAs encoding DsRed and GFP-tagged Cx43 were mixed 1:1, or in (b) 1:3 prior to transfection. Resulting plaques were homogeneously yellow (a), or consisted of yellow and green domains (b). Scale bar = 5 μm.

polypeptides, but is rather due to the strong tetramerization tendency of DsRed described above.

3.2. Rescuing DsRed-tagged Connexins

DsRed-tagged Cxs, however, can be rescued to oligomerize into functional connexons that then are transported normally to their functional site in the plasma membrane and assemble into gap junction channels and gap junction plaques when the cells were co-transfected with untagged, wild-type Cx (Fig. 2A), or GFP-tagged Cx cDNAs, respectively (Fig. 2B). Trafficking of DsRed-tagged Cxs to the plasma membrane and assembly into gap junction channel clusters was found to be proportional to the amount of co-transfected wild-type, or GFP-tagged Cx, respectively. Increasing amounts of untagged or GFP-tagged Cxs proportionally increased the number and size of assembled gap junction channel plaques, and significantly reduced the amount of aggregated Cxs within the cells (Fig. 2A). This result indicates that connexons, in which the number of DsRed-tagged Cxs is reduced to less than four (zero to three) will move to the plasma membrane, since tetramerization, and consequently precipitation of DsRed-tagged Cxs is prevented. This result further suggests that the DsRed protein can fold and develop fluorescence as a monomer, and tetramerization appears not to be required [7,10].

In our transfection experiments DsRed fluorescence was detectable approximately 12 h post transfection, both as perinuclear aggregate as well as gap junction plaques and was very bright after 16 h. This is approximately 4 h after the synthesis of detectable amounts of Cx43DsRed polypeptides by Western blot analysis (Fig. 1C). In previous studies a much slower maturation of red fluorescence was observed [7]. The difference in maturation time may be due to differences in expression systems (*E. coli* versus mammalian cells), temperature (room temperature versus 37°C), and fusion partners (polyhistidine tag versus Cx43) [7].

3.3. Co-expression of DsRed and GFP-tagged Connexins

The separation of the excitation and emission spectra of DsRed and GFP by more than 70 nm allowed an efficient discrimination of co-expressed DsRed and GFP-tagged Cxs

with standard Texas Red and FITC filter sets (Fig. 2B). Co-expressed DsRed and GFP-tagged Cxs oligomerized into mixed connexons and trafficked to the plasma membrane to assemble into gap junction channel plaques. The ratio of DsRed- and GFP-tagged Cxs within the oligomeric gap junction connexon correlated with the expression level of the two Cx chimeras. Plaques assembled from a mixture of DsRed and GFP-tagged Cx43 were either homogeneously yellow, the mixed color of red and green (equal amounts of Cx43DsRed and Cx43GFP, Fig. 2Ba), or had yellow and green domains (1:3 ratio of Cx43DsRed to Cx43GFP, Fig. 2Bb). Red plaque domains were never observed. This observation is consistent with the results obtained with co-transfected wild-type Cxs described above, and further indicates that only connexons containing less than four DsRed-tagged Cxs will traffic to the plasma membrane. Furthermore, this result demonstrates that cells do not discriminate between the GFP and the DsRed tag, and use both fusion proteins to assemble the connexon structure. GFP and DsRed monomers were found to be very similar in size and overall structure [10,11] which probably explains why cells do not select between these two protein tags.

Finally, no gap junction channel plaques were observed when DsRed-tagged Cx43 was expressed in tissue culture cells that express endogenous Cx43, such as T51B and COS7 cells that could reduce the number of DsRed-tagged Cxs per connexon. However, the amount of endogenous Cx present in these cells is very low when compared to the amount of protein translated from an expression plasmid after transient transfection. Furthermore, most of the endogenous Cx is present as gap junction channel plaques in the plasma membrane and only a very small amount is present as newly synthesized protein in the ER [19,20]. This small amount of endogenous Cx is probably not sufficient to appropriately reduce the number of the DsRed-tagged Cxs in the newly synthesized connexon precursors.

3.4. Conclusions

In this report we have described a simple method that allows the rescue of an otherwise non-functional protein complex, assembled from DsRed-tagged subunits to function by co-transfecting with a cDNA encoding untagged, or GFP-tagged subunits. This approach appears to prevent the tetramerization of the DsRed within the oligomeric complex. Furthermore, DsRed fluorescence was detected already 12 h post transfection, much earlier than previously reported, and could readily be differentiated from co-expressed GFP. Thus, this approach can eliminate the major drawbacks of this highly attractive autofluorescent protein and should be useful for many oligomeric, and maybe even non-oligomeric proteins that misbehave or aggregate when tagged with DsRed.

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