

# Genetic tags for labelling live cells: gap junctions and beyond

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The availability of green fluorescent protein (GFP) as a tracer for observing proteins in living cells has revolutionized cell biology and spurred an intensive search for GFP variants with novel characteristics, additional autofluorescent proteins and alternative techniques of protein labelling. Two recent studies – one on tagging with tetracysteine motifs and labelling with biarsenic fluorophores of different colours, and the other on GFP tagging and fluorescence recovery after photobleaching (FRAP) – show how membrane channels are added and removed from gap junctions by using different fluorescent tags to distinguish between newly synthesized and older protein populations.

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The ability to tag proteins genetically and to study their expression and functional dynamics in living cells is extremely desirable [1–3]. Green and red fluorescent proteins, additional colour variants and alternative techniques for chemically labelling proteins are now available for these purposes. These probes and techniques have been used recently to study the dynamic assembly, degradation and structural composition of gap junctions by tagging ‘connexins’ – the subunits that comprise the channel proteins of gap junctions (see Box 1 for more information on gap junctions).

In this article, fluorescent protein and alternative genetic tagging approaches are summarized, the advantages and disadvantages of the different techniques are highlighted, and recent advances in gap junction biology that have been achieved using these novel techniques for labelling live cells are described.

## GFP and GFP colour variants

Green fluorescent protein [4,5] is an excellent marker for studying the expression and functional dynamics of proteins in living cells, and literally thousands of papers have described the use of GFP for these purposes. The utility

of GFP is predominantly due to its inert nature, which is derived from its tightly packed, 11-stranded  $\beta$ -barrel structure [6,7]. The numerous potential uses of GFP quickly triggered the development of novel GFP variants with specific characteristics, such as improved fluorescence brightness, shifted excitation and emission spectra, organelle-specific targeting, reduced half-life and intrinsic properties that sense environmental changes, as well as an intensive search for other autofluorescent proteins with properties that differ from those of GFP.

Two genetically modified, fluorescence-enhanced GFP colour variants, cyan fluorescent protein (CFP) [8] and yellow fluorescent protein (YFP) [6], are particularly useful for studying simultaneous protein expression [9], protein–protein interactions [10] and localized enzyme activation [3]. Their excitation and emission spectra are far enough apart (CFP,  $Ex_{max}$  434 nm,  $Em_{max}$  477 nm; YFP,  $Ex_{max}$  514 nm,  $Em_{max}$  527 nm) to excite and to record separately the emitted fluorescence with appropriate filter sets; in addition, energy absorbed by CFP can be transferred efficiently to closely apposed (<10 nm distance) YFP chromophores, resulting in fluorescence resonance energy transfer (FRET) [2,10]. Other FRET-based approaches for studying localized enzyme activation use fluorescent labelled biosensors in combination with GFP-tagged enzymes [11].

Live-cell imaging of GFP-labelled connexins has identified the steps involved in the assembly and degradation of gap junctions [12–15] and has enabled correlation of the size of gap junctions with the number of functional channels [16]. Multicolour time-lapse microscopy of co-expressed CFP-tagged and YFP-tagged connexins has been used to investigate the structural composition of gap junctions in living cells [17]. These studies have shown that connexins are either distributed homogeneously throughout the channel cluster or segregated into well-separated

domains – a distribution that is strictly dependent on the co-expressed types of connexin [15] (Fig. 1). It has been also shown that gap junction channels and plaques are not arranged into statically packed channel arrays, as might be implied by their dense packing, but remain mobile and can undergo dynamic rearrangements in the plasma membrane [18,19] (Fig. 1).

Thus, fluorescence methods have provided novel structural data to support the earlier concept that gap junctions are dynamic structures that can be assembled and disassembled quickly in response to intracellular and extracellular stimuli. Most recently, photobleaching [20] and chemical labelling with different colours of biarsenic compounds [21] have finally shown how gap junction channels are delivered to and are removed from the channel clusters (see below) – an issue that had remained unresolved since the morphological characterization of gap junctions by freeze–fracture electron microscopy more than 30 years ago.

## Red fluorescent proteins

Proteins that fluoresce at red or far red (RFPs) wavelengths are of specific interest because eukaryotic cells and tissues have a markedly reduced autofluorescence at these higher wavelengths. This almost completely eliminates background fluorescence and so greatly increases sensitivity [22]. Despite considerable efforts, however, a red fluorescent variant of GFP has not been generated as yet. The RFP isolated from *Discosoma striata*, DsRed [23] ( $Ex_{max}$  558 nm,  $Em_{max}$  583 nm), is an obligate tetramer [24,25] that matures more slowly than GFP [26] and has a tendency to form aggregates [27].

An engineered variant of DsRed known as T1 has become available recently and efficiently solved the problem of slow maturation [28], and aggregation has been reduced by genetic engineering in the commercially available DsRed variant DsRed2 [29], but tetramerization can interfere severely with the behaviour of host proteins. This has been observed with

### Box 1. Gap junctions and direct cell to cell communication

Gap junction channels are ubiquitously distributed, double-membrane protein structures that create hydrophilic pores across the membranes [a]. They are important in the coordination of development, cellular homeostasis and tissue function. The channels mediate direct cell to cell communication by allowing the passage of small biological molecules ( $\leq 1$  kDa) from one cell to another [b]. Gap junction channels cluster together to create characteristic two-dimensional arrays of individual channels, termed 'gap junction plaques', that are structurally distinguishable from other clustered arrangements of proteins present in the plasma membrane. Fewer than ten to many thousands of individual channels can be combined in a single plaque, which can extend from several nanometres to several micrometres in diameter [b] (Fig. 1).

In mammals, gap junction channels comprise two half-channels, termed 'connexons', that are each provided by one of the two neighbouring cells. Two connexons dock in the extracellular space between two adjoining plasma membranes to form the complete, double-membrane intercellular junction. Each connexon comprises six polytopic transmembrane protein subunits termed connexins (Fig. 1). In humans there are up to 20 connexin isoforms [c]. These represent structurally conserved nonglycosylated members of a multigene family that traverse the membrane four times and differ mainly in the length and characteristics of their intracellular and carboxy-terminal domains.

Studies have shown that connexins are cotranslationally integrated into the endoplasmic reticulum membrane and then assembled into hexameric connexons, and that they pass through the Golgi apparatus to reach the plasma membrane [d]. Although the different connexin isoforms have a distinct tissue distribution, many cell types express more than one isoform of connexin. This allows the assembly of both homo-oligomeric connexons, and hetero-oligomeric connexons that are constructed from different connexin isoforms. Furthermore, in addition to homodimeric (= homotypic) channels, heterodimeric (= heterotypic) channels comprising two different types of connexon can be assembled between adjacent cells expressing different connexin isoforms [b, d]. It seems obvious that connexin protein expression, intracellular trafficking, and gap junction channel assembly and degradation are important steps in the biology of this structure that have to be coordinated and regulated precisely to ensure proper function.

Mutations that cause severe pathological disorders, including neuropathies, lens cataracts, deafness and several complex dermatological disorders, have been identified in different connexin isoforms [e-g]. The deletion of specific connexin genes by targeted ablation, and analysis of connexin expression, has increased our knowledge of gap junction function [c]; however, in only a few cases has it led to recognition of the mechanism that causes the disease or to a potential model of

pathogenesis. Alternative approaches, such as investigating the biosynthesis, structural composition and function of gap junctions in living cells, as reviewed briefly in this article, will help us to understand the complex function of gap junctions under healthy and pathological conditions.

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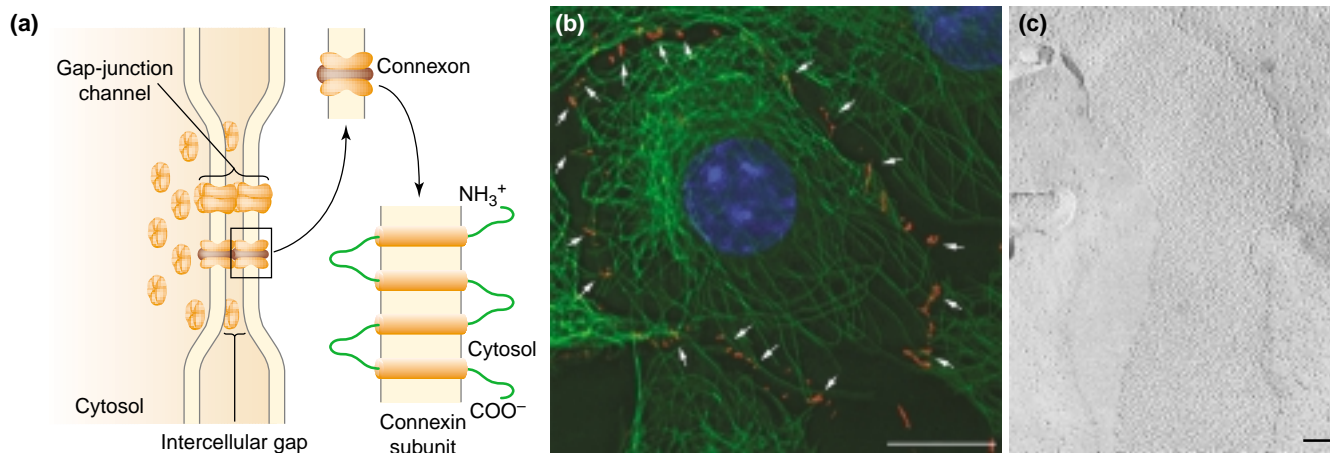


Fig. 1. The structure of gap junctions. (a) Representation of a gap junction, gap junction channels, the half-channel or connexon, and the subunit proteins, connexins. (Modified, with permission, from Ref. [h].) (b) Fluorescence image of

rat epithelial T51B cells with gap junctions (labelled in red) in the adjoining plasma membranes (indicated by arrows) identified by labelling with antibodies specific to connexin 43. The cell nucleus (labelled in blue) and microtubules (labelled in green) are also shown.

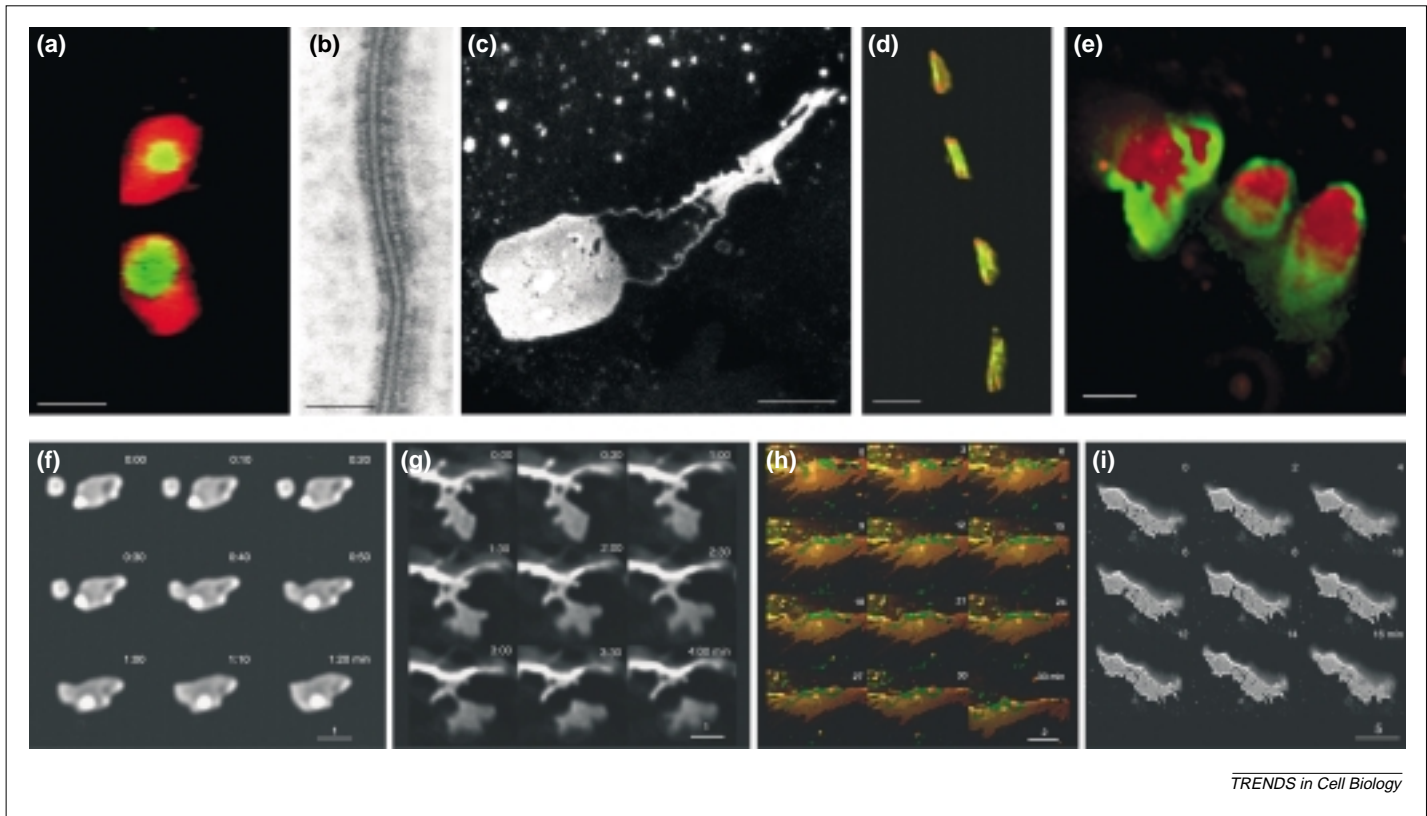
Bar, 10  $\mu$ m. (c) Ultrastructure of a gap junction obtained by freeze–fracture electron microscopy. Individual, densely packed channels, with an approximate centre-to-centre spacing of 10 nm, are visible. Bar, 0.1  $\mu$ m.

DsRed-tagged connexins, which failed to assemble into gap junctions and remained in a perinuclear region [30]. The DsRed-tagged connexins could be rescued, however, by the co-expression of untagged or GFP-tagged connexin, which assembled

efficiently into gap junctions possibly because the number of DsRed-tagged connexins in a hexameric connexon was reduced to fewer than four [30].

Very recently, exciting progress has been made in suppressing

RFP oligomerization and in developing monomeric RFPs. Two identical RFPs have been linked covalently and expressed as a non-oligomerizing fusion tag ([31]; and A.F. Fradkov *et al.*, pers. commun.). Site-directed and random mutagenesis of



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Fig. 1. High-resolution micrographs acquired in living cells of gap junction channels assembled from connexins with different genetic tags. (a) Two gap junction plaques assembled from connexin 43 tagged with a tetracysteine peptide. Older channels in the centre are labelled with FIAsh (green) and newly synthesized channels, which have added to the outer plaque margins, are labelled with ReAsH (red). Bar, 2  $\mu\text{m}$ . (b) A connexin 43 gap junction plaque labelled with ReAsH and processed for electron microscopy using DAB photoconversion, which results in the typical appearance of a gap junction viewed on its edge. Bar, 0.1  $\mu\text{m}$ . (c) A gap junction plaque assembled from GFP-tagged connexin 43 in which the fluorescence of a central area (appearing dark) has been photobleached permanently. Newly synthesized channels that have added to the cluster within an hour are visible as fluorescent rims lining the outer margin of the bleached area. Constitutive transport vesicles and degradative vesicular structures are also visible in the vicinity of the plaque. Bar, 2  $\mu\text{m}$ . (d) A third approach to differentiate between older and newer channels is based on the successive expression of GFP- and DsRed-tagged connexins. GFP-tagged connexin 43 is expressed in inducible, stably transfected HeLa cells; these cells are then transiently transfected with a mixture of DsRed-tagged and wild-type connexin 43 cDNAs, and further expression of GFP-tagged connexin 43 is halted. Again, newly synthesized channels are added as a red fluorescent rim around the central GFP-tagged channels. Bar, 2  $\mu\text{m}$ . (e) Clusters of gap junction channels assembled from two different types of connexin. Channels assembled from CFP-tagged connexin 43 (green) are added around a central core of YFP-tagged connexin 26 channels (red). Bar, 2  $\mu\text{m}$ . (f–i) Time-lapse microscopy of gap junctions assembled from GFP-tagged connexin 43 (f, g, i), and CFP-tagged connexin 43 (green) and YFP-tagged connexin 26 (red) (h) in transfected HeLa cells shows that the arrangement of gap junction channels is mobile. This can result in plaque fusion (f), plaque splitting (g), and directional (h) and distorted (i) flow in the plane of the membranes. In (h), note how domains comprising CFP-tagged connexin 43 fuse and split but never mix with the domains containing YFP-tagged connexin 26. (a) and (b) were reproduced, with permission, from [21], (c) from [20], (e) from [15] and (f–i) from [18]. Scale bars in parts (f–i) are given in micrometres.

a new RFP isolated from *Heteractis crisper*, HcRed, has facilitated the generation of a dimeric, far-red-shifted HcRed variant, HcRed-2A ( $\text{Ex}_{\text{max}}$  588 nm,  $\text{Em}_{\text{max}}$  618 nm) [32], which also promises the generation of a monomeric HcRed variant (S. Lukyanov, pers. commun.). Finally, site-directed and evolutionary mutagenesis has resulted in the generation of a fast-maturing monomeric DsRed derivative, mRFP1 ( $\text{Ex}_{\text{max}}$  584 nm,  $\text{Em}_{\text{max}}$  607 nm) [31], that allows the assembly of functional gap junctions when fused to the C-terminus of connexin 43 [31]. But as Campbell *et al.*

state [31], mRFP1 is not ideal for all applications because its fluorescence quantum yield and extinction coefficient (0.25 and 44 000  $\text{M}^{-1} \text{cm}^{-1}$ , respectively) are significantly lower than those of other DsRed variants. But further mutagenesis promises the development of very useful, fast-maturing monomeric RFPs in the near future.

**Tetracysteine peptides labelled with biarsenic fluorophores**  
Because of the limited availability of fluorescent proteins and colour variants,

and because known fluorescent proteins are fairly large [25–27 kDa ( $\sim 24 \times 42 \text{ \AA}$ )] and cannot be reduced substantially in size [33], there is keen interest in alternative ways of genetically labelling proteins. Such an alternative approach that requires only the genetic attachment or insertion of a short peptide of 6–20 amino acids containing a tetracysteine motif (Cys-Cys-Xaa-Xaa-Cys-Cys, where the central Xaa-Xaa is preferentially Pro-Gly [34]) was described a few years ago [35].

Cells expressing the nonfluorescent, peptide-tagged protein are exposed to a small (700 Da), membrane-permeant, nonfluorescent biarsenic derivative of fluorescein, termed FIAsh-EDT<sub>2</sub> (for fluorescein arsenical helix binder bound to two ethanedithiol molecules). In the presence of the tetracysteine motif the two EDT molecules are released. FIAsh then binds with high affinity and specificity (the effective dissociation constant of FIAsh-peptide complexes is about  $10^{-11} \text{M}$ ) to the tetracysteine motif, thereby becoming strongly fluorescent at green wavelengths ( $\text{Ex}_{\text{max}}$  508 nm,  $\text{Em}_{\text{max}}$  528 nm) [35,36]. Unbound excess FIAsh-EDT<sub>2</sub> is removed to detect the labelled proteins. Griffin *et al.* state [35,36] that endogenous competing proteins or ligands that would bind FIAsh are rare enough to permit the easy detection of

**Table 1. Advantages and disadvantages of autofluorescent protein and biarsenical tetracysteine-peptide tags such as FIAsh and ReAsH<sup>a</sup>**

	Biarsenical peptide tags	Autofluorescent protein tags <sup>b</sup>
Tag size	Small, short peptides, 6–20 amino acids long [21,34,35].	Large, 25–27 kDa proteins, can-shaped, ~24 × 42 Å in size [6,7,24,25].
Tolerance	In general, probably well tolerated owing to small tag-size.	GFP and GFP-variants are well tolerated; however, problems occur with DsRed owing to tetramerization and aggregation. RFPs from other species [32] and genetically altered monomeric [31] and dimeric [32] derivatives might resolve these problems
Detectability	Tagged proteins become visible after staining with biarsenic compounds and washing; labeling is possible in living cells [34,36].	Tagged proteins are readily visible. Wild-type RFPs can require hours to mature and become fluorescent [26], however, rapidly maturing derivatives have been generated [28]. Certain FPs require specific lasers and filter sets for excitation and detection.
Sensitivity	Less sensitive than GFP (several μM) [34].	Highly sensitive (<1 μM) [38].
Colors	Red, green, and blue biarsenic compounds available; Potentially more colors possible [34].	Limited number of useful proteins and color variants available; new FPs and variants have been introduced.
Brightness	FIAsh and ReAsH have comparable brightness to GFP (quantum yield: 0.1–0.6) [34].	Limited number of FPs and variants are sufficiently bright (quantum yield: 0.2–0.8) [34].
Live-cell imaging	Time-lapse imaging predicted to be possible.	Time-lapse imaging possible, CFP bleaches faster than GFP, YFP and DsRed [9,17].
Photobleaching	Good photobleach resistance, permanent photobleaching for FRAP experiments predicted to be possible [34].	Low to very high photobleach resistance, permanent photobleaching for FRAP experiments possible, DsRed more difficult to photobleach [2].
Simultaneous labeling	Different proteins cannot be labeled simultaneously; tetracysteine-motifs will be recognized by all biarsenic compounds added; in combination with FP-tags possible.	Simultaneous labeling of two and more proteins possible.
FRET	FRET only possible between biarsenic chromophores and FPs.	Good FRET donors and acceptors [10,11].
Toxicity	Authors argue that biarsenic compounds are not toxic for operator because arsenics are protected by EDT [34,36].	Not toxic for operator.
Ultrastructural analysis	ReAsH allows imaging of structures in the same cell by both fluorescence and electron microscopy [21].	No direct imaging of FP-tagged proteins possible by electron microscopy.
Other uses	Fluorescence remains after denaturing gel electrophoresis; Useful for fluorescence polarization assays; tetracysteine tag usable for affinity chromatography [34].	Autofluorescence does not remain after denaturing gel electrophoresis; limited use for fluorescence polarization assays; purification requires immobilized FP-specific antibodies [34].

<sup>a</sup>FIAsh and ReAsH will become available commercially in 2002 from PanVera.  
<sup>b</sup>Abbreviations: EDT, ethanedithiol; FPs, fluorescent proteins; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GFP, green-fluorescent protein; RFPs, red-fluorescent proteins.

recombinant proteins over background. In addition, FIAsh-peptide complexes are predicted to remain stable for days in the absence of excessive (millimolar) concentrations of competing EDT [35].

Recently, blue and red fluorescent analogues of FIAsh have been synthesized [34], of which the red analogue – a biarsenic derivative of resorufin termed ReAsH (for resorufin arsenical helix binder;  $E_{x_{max}}$  593nm,  $E_{m_{max}}$  608 nm) – has been used in combination with FIAsh to investigate how gap junction channels are added and removed from the clusters of gap junction channels [21]. Like FIAsh, ReAsH has very little fluorescence when bound to EDT but becomes brightly fluorescent on binding to tetracysteine motifs. In analogy to previous experiments using GFP and derivatives [12,15], a tetracysteine peptide of 17 amino acids was

incorporated genetically at the C-terminal tail of connexin 43 (the most widely distributed subunit protein of gap junction channels) and expressed in HeLa cells, which do not express endogenous connexins. The population of recombinant connexin 43 was labelled with FIAsh-EDT<sub>2</sub>, excess FIAsh-EDT<sub>2</sub> washed away and, after further incubation, the cells were relabelled with ReAsH-EDT<sub>2</sub> to identify newly synthesized channels.

Using this procedure, Gaietta *et al.* [21] have shown that newly synthesized channels (labelled red with ReAsH) are added as rings around a central core of older channels (labelled green with FIAsh; Fig. 1a). Incubating the cells for longer periods before relabelling them with ReAsH showed that the central core of old channels becomes smaller, which indicates that channels are replenished

continuously by new additions to the outer cluster margin while older channels are removed simultaneously from the plaque centre.

An interesting additional property of ReAsH is that it can be used after fixation to catalyse a highly localized photoconversion of diaminobenzidine (DAB) into an insoluble precipitate that is visible by electron microscopy and allows a direct correlation of fluorescence and electron microscopy images [21] (Fig. 1b). As Gaietta *et al.* [21] state, previously such correlations required the diffusion of antibodies, protein toxins or antisense oligonucleotides into the fixed tissue, or were achieved partially by subsequent labelling with immunogold. Neither FIAsh, nor any known fluorescent protein, is capable of photooxidizing DAB.

### GFP and FRAP

Lauf *et al.* [20] have used an alternative approach based on GFP-tagged connexins and fluorescence recovery after photobleaching (FRAP) [2] to visualize the addition and removal of channels to gap junction plaques. They photobleached permanently the GFP fluorescence of defined areas of gap junction plaques by means of a confocal microscope laser and then followed the addition of newly synthesized channels to the plaque in living cells over time.

As found by Gaietta *et al.* [21], newly synthesized channels are added homogeneously to the outer plaque margins, which are visible as a fluorescent rim that lines the central area of photobleached channels (Fig. 1c). Size measurements indicated that the plaques were not growing but were in a steady state of outer channel accretion and central channel depletion [20]. In addition, by applying multicolour time-lapse microscopy to study intracellular connexin trafficking, as well as FRAP and fluorescence loss in photobleaching (FLIP) [2] experiments to determine the dynamics of plasma membrane fluorescence, Lauf *et al.* [20] could show further that connexons are delivered predominantly to the nonjunctional plasma membrane surface, thus generating a plasma membrane pool of connexons that can move laterally in the membrane to reach gap junction plaques.

### Single-chain antibodies

An approach related to the chemical conversion of biarsenic compounds uses single-chain antibodies to target a fluorophore to a cellular compartment or organelle. A targeting sequence that is specific for the cellular compartment or organelle is fused to the cDNA of a single-chain antibody. After expression in cells, the targeting sequence will deliver the fusion protein to its target site. Cells are then incubated with a membrane-permeable, fluorescence-tagged peptide sequence with high affinity for the antibody, which will result in specific labelling of the structure. This approach has the advantage of allowing the targeting of chemical probes with custom-designed spectral and indicator properties to different cellular compartments – such as the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, chloroplasts, lysosomes or the plasma

membrane – for which specific targeting sequences are available [37].

Farinas and Verkman [37] have used their novel technique to label and to target marker proteins to the endoplasmic reticulum, Golgi and plasma membrane of living Chinese hamster ovary cells, and to study pH regulation in the Golgi apparatus.

### Concluding remarks

The availability of GFP and the tremendous progress in developing brightly fluorescent GFP variants with shifted excitation and emission spectra, in the cloning and genetic improvement of red-shifted and far-red-shifted fluorescent proteins, and in the development of novel approaches for chemically labelling proteins have provided us with many different experimental techniques based on fluorescence tagging. These techniques can be used in both high-throughput and single-cell approaches and will greatly enhance our understanding of protein expression, localization, interaction and dynamics. Improved and novel imaging, as well as computational approaches, will further foster these endeavours.

Using these live-cell labelling techniques in gap junction biology has not only provided us so far with a novel view of the structural composition and dynamics of gap junctions but also provided a mechanistic insight into how such complex, multi-unit structures are assembled and turned over. Features such as the small size of the tag, the fast kinetics of labelling, the potential to custom design many suitable colour variants and the ability to obtain fluorescence, biochemical and ultrastructural information from the same cells [21] make chemical probes such as FAsH and ReAsH promising alternatives to autofluorescent proteins (see Table 1 for a comparison of advantages and disadvantages of both techniques). Future experiments, however, will have to prove the versatility and general practicability of these techniques.

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## Human Sir2 and the 'silencing' of p53 activity

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Members of the evolutionarily conserved silent information regulator 2 (Sir2) protein family are nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent histone deacetylases. In yeast, the founding Sir2 protein is known to function in transcriptional silencing processes through the deacetylation of histones H3 and H4, thus setting up a repressive chromatin structure. Yeast and *Caenorhabditis elegans* Sir2 are also involved in regulating the life span of these organisms. Until recently, the function of mammalian Sir2 family members was completely unknown. However, several recent studies have now determined a remarkable function for the human SIRT1 protein, which is the closest human homolog of yeast Sir2. SIRT1 specifically associates with the p53 tumor suppressor protein and deacetylates it, resulting in negative regulation of p53-mediated transcriptional activation. Importantly, p53 deacetylation by SIRT1 also prevents cellular senescence and apoptosis induced by DNA damage and stress.

Post-translational histone modifications play a critical role in the regulation of eukaryotic gene expression. The highly conserved N-terminal tails of histones H3 and H4 contain several lysine residues that are acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases

(HDACs). In general, histone acetylation is associated with high levels of transcription and deacetylation is associated with transcriptional repression. This notion corresponds well with multiple examples of HATs that are found in coactivator complexes and HDACs that are components of corepressor complexes. However, it has become increasingly clear that non-histone proteins, including many transcription factors, are also acetylation targets for HATs [1]. So widespread is protein acetylation that it might eventually be found to rival phosphorylation in its importance within cellular signaling cascades. Recent exciting studies now implicate the silent information regulator 2 (Sir2) family of deacetylases as major players in this niche [2–5], thus extending the role of Sir2 beyond heterochromatic silencing.

The emergence of Sir2 as a histone deacetylase  
The HDACs are divided into three classes based on sequence homology [6]. Class I enzymes have high homology to the yeast Rpd3 deacetylase and the class II enzymes are homologous to the yeast HDA1 deacetylase. The third class of HDACs are the Sir2 family of enzymes. *SIR2* from the budding yeast *Saccharomyces cerevisiae* was the founding member of this highly conserved gene family, and was identified

as a gene required for transcriptional silencing at the *HM* silent mating-type loci [7]. Related proteins have been identified in Archea, eubacteria, fungi, and metazoans, including humans [8]. The number of Sir2-like proteins in each species varies. For example, in addition to Sir2 itself, *S. cerevisiae* has four other family members named Hst1 through Hst4 [8]. Human cells contain eight different family members named SIRT1 through SIRT8 [9]. The Sir2 proteins were only recently shown to have histone deacetylase activity and their distinguishing feature is that they rely completely on the oxidized form of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) for activity [10–12]. NAD<sup>+</sup> is a crucial electron-accepting cofactor used in multiple oxidation reactions, including many related to energy utilization.

The critical role of yeast Sir2 in transcriptional silencing is well established so the fact that Sir2 is a histone deacetylase makes a lot of sense. Why, however, does Sir2 HDAC activity require NAD<sup>+</sup> as a cofactor? It turns out that Sir2 hydrolyzes NAD<sup>+</sup> as part of the deacetylation reaction, resulting in nicotinamide and *O*-acetyl-ADP-ribose products [7]. The acetyl group is removed from a lysine residue and transferred to the 2' or 3' hydroxyl group of the ribose ring of ADP-ribose [13,14]. The hydrolysis of NAD<sup>+</sup> is therefore coupled directly to the