Biosynthesis and structural composition of gap junction intercellular membrane channels

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Autofluorescent reporter technology – connexins – deconvolution (DV) microscopy – green fluorescent protein (GFP) – membrane proteins

Gap junction channels assemble as dodecameric complexes, in which a hexameric connexon (hemichannel) in one plasma membrane docks end-to-end with a connexon in the membrane of a closely apposed cell to provide direct cell-to-cell communication. Synthesis, assembly, and trafficking of the gap junction channel subunit proteins referred to as connexins, largely appear to follow the general secretory pathway for membrane proteins. The connexin subunits can assemble into homo-, as well as distinct hetero-oligomeric connexons. Assembly appears to be based on specific signals located within the connexin polypeptides. Plaque formation by the clustering of gap junction channels in the plane of the membrane, as well as channel degradation are poorly understood processes that are topics of current research. Recently, we tagged connexins with the autofluorescent reporter green fluorescent protein (GFP), and its cyan (CFP), and yellow (YFP) color variants and combined this reporter technology with single, and dual-color, high resolution deconvolution microscopy, computational volume rendering, and time-lapse microscopy to examine the detailed organization, structural composition, and dynamics of gap junctions in live cells. This technology provided for the first time a realistic, three-dimensional impression of gap junctions as they appear in the plasma membranes of adjoining cells, and revealed an excitingly detailed structural organization of gap junctions never seen before in live cells. Here, I summarize recent progress in areas encompassing the synthesis, assembly and structural composition of gap junctions with a special emphasis on the recent results we obtained using cell-free translation/membrane-protein translocation, and autofluorescent reporters in combination with live-cell deconvolution microscopy.

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Definitions and Abbreviations. Currently, two different nomenclatures are in use to name connexins. One groups the connexins into α and β subclasses, based on distinct amino acid sequence similarities, the other names the connexins according to their molecular mass in kDa. A fusion of both nomenclatures, e.g. α_1(Cx43), β_1(Cx32), and β_2(Cx26) is used in this review, as was suggested on the recent International Gap Junction Conference (28 August–2 September 1999, Gwatt, Switzerland), until a standardized nomenclature is accepted.
CFP Cyan fluorescent protein. – Cx Connexin. – DV Deconvolution. – ER Endoplasmic reticulum. – GFP Green fluorescent protein. – TM Transmembrane. – YFP Yellow fluorescent protein.

Introduction

Gap junctions are described as distinct structures in the adjoining plasma membranes of contacting cells with an almost ubiquitous distribution in pluricellular organisms. Extensive ultrastructural analyses, in particular by freeze-fracture electron microscopy (see related article by Benedetti et al. (2000), this issue) revealed that gap junctions are aggregates of tightly packed channels that cluster together to create characteristic two-dimensional sheets of channels, or plaques, that are structurally distinct from other clustered arrays of particles also present in the plasma membrane (McNutt and Weinstein, 1970; Friend and Gilula, 1972; Rash et al., 1974). Typically, less than a hundred to many thousand individual channels are combined into a single plaque that can extend from less than a hundred nanometers to several micrometers in diameter (McNutt and Weinstein, 1970). Further analysis has shown that gap junction channels represent double membrane protein structures that create hydrophilic pores across the membranes (Makowski et al., 1977). A high-resolution three-dimensional structure of a recombinant gap junction channel resolved to 7.5 Å is now available (Unger et al., 1999).

Gap junction channels mediate direct cell-to-cell communication by allowing the passage of nutrients, metabolites, and small biological molecules, such as second messengers up to approximately 1 kDa in size from one cell to the other (Gilula et al., 1972). Many reports have shown now that gap junctional cell-to-cell communication plays a crucial role for the normal function of cells in developing and differentiated tissues and

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organs that has been supported by the observation that impaired gap junctional communication, often correlating with mutations in the constituent proteins, associates with various pathological disorders including cardiac arrhythmia, tumorigenesis, Charcot-Marie-Tooth X-linked neuropathy, cataractogenesis, and sensorineural deafness (recently reviewed in Simon and Goodenough, 1998; Simon, 1999; White and Paul, 1999).

In mammals, gap junction channels have been characterized to be composed of two hemi-channels, termed connexons, each provided by one of the two neighboring cells. Two connexons dock end-to-end in the adjoining plasma membranes to form the complete, double-membrane intercellular channel. Electron microscopy in combination with image processing (Unger et al., 1999), hydrodynamic analysis (Musil and Goodenough, 1993; Kistler et al., 1994; Cascio et al., 1995; Falk et al., 1997), and chemical crosslinking (Cascio et al., 1995; Musil and Goodenough, 1993; vanSlyke and Musil, 2000) have indicated that each connexon is composed of 6 polytopic transmembrane protein subunits, termed connexins (Cx). Partial crosslinks that indicated a pentameric structure of connexons that have been seen with α_1(Cx43) were recently attributed to anomalously fast migrating fully crosslinked α_1(Cx43) hexamers (VanSlyke and Musil, 2000). Only a single type of structural protein is therefore required, and is sufficient, to construct gap junction channels. Hitherto, at least 14 different connexin subtypes have been cloned and sequenced from mice. All represent structurally conserved nonglycosylated members of a multigene family 25 to 50 kDa in size that mainly differ in the length of their C-terminal domain. Although the different connexin isotypes exhibit a distinct tissue distribution, many cell types express more than one connexin isotype. This may allow the assembly of hetero-oligomeric connexons constructed from different connexin isotypes, in addition to the assembly of homo-oligomeric connexons constructed from a single connexin isotype (see (Kumar and Gilula, 1996) for a recent structural review). It appears obvious that connexin protein synthesis and assembly, intracellular trafficking, channel formation, and degradation are important steps in the biosynthesis of gap junctions that have to be coordinated and regulated precisely to enable proper channel function. Here, I summarize recent progress in areas encompassing the synthesis, assembly and structural composition of gap junctions.

Synthesis of connexins, maturation into gap junction channels, and gap junction degradation

The translocation of proteins across membranes and their intracellular trafficking represent fundamental processes which mechanisms and pathways appear to be basically identical in all eukaryotic cells (reviewed in Hurtley and Helenius, 1989; Schatz and Dobberstein, 1996). The delineation of the steps involved in the synthesis, assembly and degradation of connexin polypeptides and gap junctions is schematically depicted in Figure 1.

The membrane integration process of connexins was primarily investigated using cell-free translation systems supplemented with endoplasmic reticulum (ER)-derived membrane vesicles (microsomes) (see (Falk, 2000 a, b) for methodology papers). Studies from several laboratories, including our own, have demonstrated that connexins are synthesized at the ER membrane in a process that involves signal recognition particle (SRP), an internal signal anchor (SA) sequence, docking of the nascent-chain/ribosome complex to the translocon, and co-translational integration of the connexin polypeptides into the ER membrane (Falk et al., 1994; Zhang et al., 1996; Falk and Gilula, 1998) (Figure 1, step 1). Similar processes have been described for other transmembrane proteins, as well as secretory proteins. N-glycosylation, and protease protection assays demonstrated that the final, functional transmembrane topology of the connexin polypeptides with four transmembrane domains, two extracellular loops, and cytoplasmically located amino- and carboxyl termini is achieved during their ER membrane integration (Falk et al., 1994; Falk and Gilula, 1998) (Figure 1, step 1).

Since gap junction channels are oligomeric protein structures the connexin subunits have to assemble before they can function. Using an integrated biochemical and biophysical analysis we, and others, have observed functional assembly of gap junction connexons composed of α_1(Cx43), or β_1(Cx32) in isolated ER membrane vesicles (microsomes) following translation/membrane translocation in a cell-free translation system (Falk et al., 1997; Ahmad et al., 1999) (Figure 1, step 2; Figure 2). Oligomerization of connexin polypeptides within the ER is further supported by the detection of gap junctions present in the ER membranes of cells expressing recombinant connexins (Kumar et al., 1995), by the observations of George et al. (1998; 1999) who suggested oligomerization to occur in a specialized region of the ER, the intermediate compartment or ERGIC (Tartakoff, 1986; Schweizer et al., 1990), and by the preliminary results obtained by analyzing connexin oligomerization in liver tissue (A. Kehlenbach, M. M. Falk, and N. B. Gilula, unpublished observations). Assembly of α_1(Cx43) expressed endogenously in Normal Rat Kidney (NRK), and Chinese Hamster Ovary (CHO) cell lines was also reported to occur later, after exit from the ER in late Golgi membranes (Musil and Goodenough, 1993) (alternative ER-Golgi trafficking depicted with black arrows in Figure 1). In a recent publication the authors, however, acknowledge that their observation may have been a cell type-specific (VanSlyke and Musil, 2000). Recent results obtained in the author's laboratory by studying the assembly and intracellular transport of connexin-fusion proteins indicates that assembly in the ER may indeed be a necessary prerequisite for the further transport of connexons through the secretory pathway (M. Falk, manuscript in preparation). This has been shown for most known oligomeric membrane proteins, including voltage- and ligand-gated ion channel subunits (see Green and Millar, 1995 for review) that are structurally similar to connexins.

Subcellular fractionation, immunoprecipitation, and immuno-colocalization in combination with antibodies directed against subcellular compartment marker proteins that were performed in various tissues (De Sousa et al., 1993; Rahman et al., 1993; Falk et al., 1994), as well as tissue culture cell lines expressing endogenous (Musil and Goodenough, 1991; Laird et al., 1995) or recombinant connexins (Kumar et al., 1995; Deschenes et al., 1997; Falk, manuscript submitted), repeatedly detected connexin polypeptides in the ER, the Golgi, and in the plasma membranes, indicating that the connexins are transported by successive vesicle budding and fusion from the ER through the Golgi stacks to the plasma membrane following the general intracellular transport route referred to as secretory pathway (reviewed in Pfeffer and Rothman, 1987) (Figure 1, steps 1–5). Furthermore, no gap junction channel assembly and/or gap junction plaque formation was observed

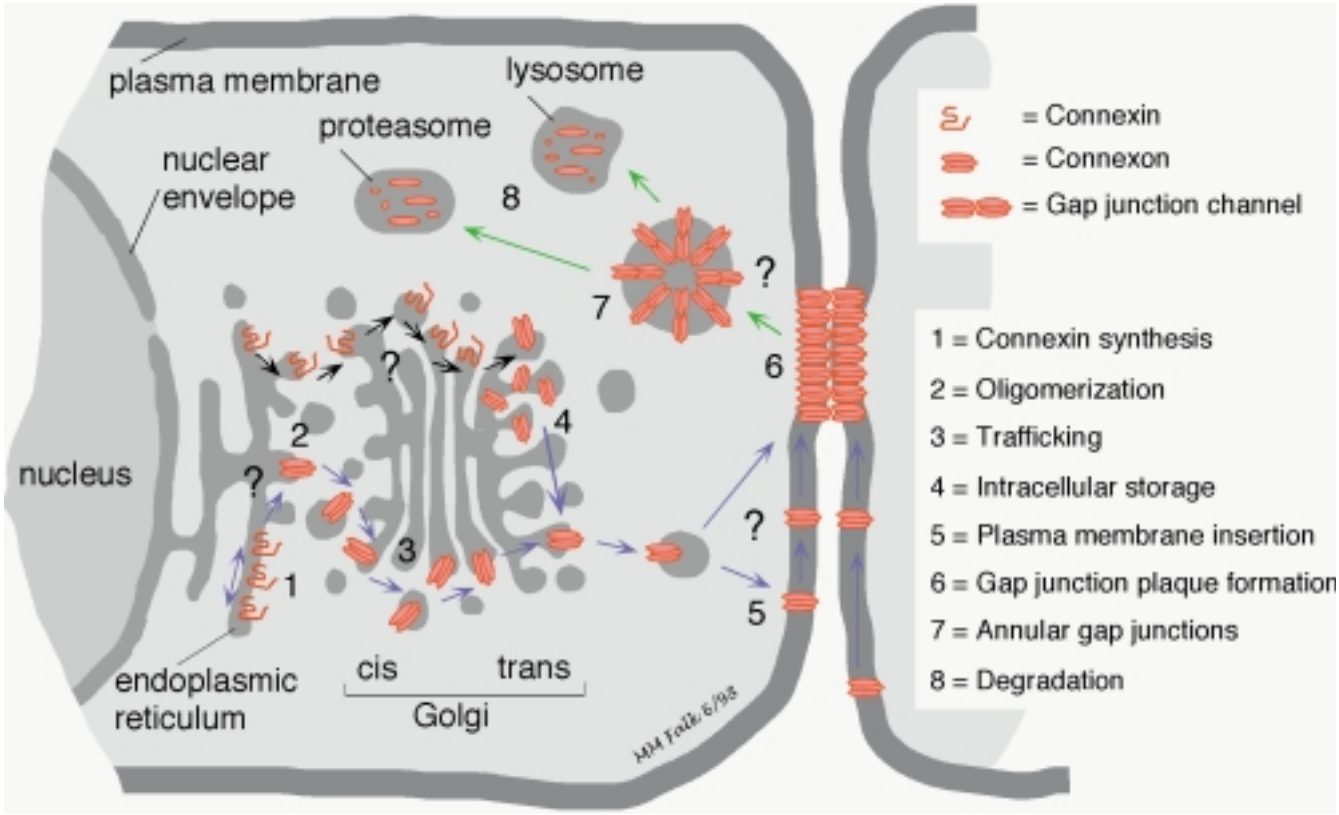

Fig. 1. Schematic representation of the synthesis, assembly, and degradation of gap junction membrane channels based on cell-free, in vitro, and in vivo analyses. Connexin synthesis at the endoplasmic reticulum membrane (1), oligomerization into gap junction connexons (hemichannels) (2), trafficking along the secretory pathway (3), intracellular storage within the Golgi apparatus (4), insertion of the gap junction connexons into the plasma membrane (5), clustering of many individual gap junction channels into a plaque (6), proposed internalization of the plaque via formation of annular gap junctions (7), and degradation of gap junction channels via lysosomal and/or proteasomal pathways (8), are marked as steps 1 to 8. Some areas of current discussion have been highlighted with *question marks*. *Blue arrows* indicate synthesis and assembly in the endoplasmic reticulum membrane. *Black arrows* indicate less likely transport of unassembled connexins to the trans side of the Golgi apparatus. *Green arrows* indicate degradation pathways of gap junction channels. Reproduced from Yeager et al. (1998) with kind permission of Elsevier Science Ltd.

in cells that were treated with drugs known to interfere with the secretory pathway, such as brefeldin A (BFA), and carbonyl cyanide m-chlorophenylhydrazone (CCCP), or were kept at non-permissive temperature (De Sousa et al., 1993; Musil and Goodenough, 1993; Laird et al., 1995). Studies performed by George et al. (1999) further suggest that connexins, especially β_2(Cx26) may use in addition alternative, not yet described trafficking pathways.

How gap junction connexons are directed to, and localized in the plasma membrane is not well understood, but is the topic of intense current research in the author's, as well as in other laboratories. In principal, two different pathways are thinkable. Either, the connexons are transported directly to the junctional site, or they are inserted somewhere else into the plasma membrane, and then are transported by lateral movement to the junctional site (Figure 1, step 5). Preliminary results in the author's laboratory obtained by imaging the assembly of gap junctions in non-polarized live tissue-culture cells by time-lapse microscopy (see below; Falk, manuscript in preparation) favor the latter hypothesis. This view is further supported by the presence of gap junction connexons (also termed hemichannels) in the plasma membranes of cultured cells (Li et al., 1996; Quist et al., 2000). After the delivery of assembled connexons to the plasma membrane, the hemichannels in the membranes of apposed cells are believed to register and pair via interactions of the extracellular loop domains of the connexin subunits, forming the complete dodecameric intercellular channels that then aggregate into gap junction plaques (Figure 1, steps 5–6). This process is enabled, or at least facilitated by calcium-dependent cell-adhesion molecules such as E-cadherins (Musil et al., 1990; Jongen et al., 1991; Meyer et al., 1992; Fujimoto et al., 1997; reviewed in Laird, 1996; Yeager et al., 1998). A linkage between α_1(Cx43) with the cytoskeletal proteins ZO-1 and α-spectrin was recently described (Toyofuku et al., 1998; Giepmans and Moolenaar, 1998; Jin et al., 2000). A linkage between α_1(Cx43) polypeptides and ZO-1 implies that distinct elements of the cytoskeleton are involved in certain aspects of gap junction function. However, not all connexin polypeptides have a ZO-1-binding site, and yet unpublished results demonstrate that the loss of the ability to bind ZO-1 does not interfere with the normal assembly, trafficking, and localization of gap junction plaques (B. N. Giepmans, M. M. Falk, unpublished results). Future research may soon elucidate the biological function of ZO-1 binding to connexins.

The degradation and turnover of gap junctions is still a poorly understood process. Connexins appear to be turned over rapidly within hours (Yancey et al., 1981; Fallon and Goodenough, 1981; Laird et al., 1991; Beardslee et al., 1998), a surprising result for a structural protein that forms a gateable channel structure. It may indicate that the currently proposed function of gap junctions is quite incomplete. Gap junction degradation is further complicated by the observations that gap junction channels, once formed, cannot be separated again into hemichannels under physiological conditions (Goodenough and Gilula, 1974; Ghoshroy et al., 1995). Double-membrane vesicular structures that contain densely packed complete dodecameric gap junction channels, termed annular gap junctions (Figure 1, step 7), were observed by electron microscopic studies in the cytoplasm of cells connected via gap junctions. They were interpreted to represent the initial degradation products of gap junctions formed by the internalization of complete or large fragments of gap junction plaques (Larsen et al., 1979; Mazet et al., 1985; Severs et al., 1989). Complete gap junction degradation finally appears to involve lysosomal, as well as proteasomal pathways (Rahman et al., 1993; Ginzberg and Gilula, 1979; Risinger and Larson, 1983; Laing and Beyer, 1995; reviewed in Laird, 1996; Spray, 1998; Yeager et al., 1998) (Figure 1, step 8).

Assembly of connexins into gap junction connexons

As illustrated above, assembly of connexins into gap junction channels represents a complicated process. Homo-oligomeric connexons (hemichannels) composed of only one connexin isotype are believed to exist in vivo since several cell types express only one known connexin isotype. Support for the presence of homo-oligomeric connexons has been provided by structural analyses of individual gap junction plaques (Risek et al., 1994; Fujimoto et al., 1997; Ko et al., 1999), by the assembly of gap junctions in cultured cells that are structurally identical to gap junctions assembled in vivo after the expression of a single connexin isotype in baculovirus-infected insect cells (Stauffer et al., 1991; Buehler et al., 1995), in transfected tissue culture cells (Elfgang et al., 1995; Kumar et al., 1995; Unger et al., 1999), or in cell-free translation systems that were supplemented with ER-derived membranes (Falk et al., 1997; Ahmad et al., 1999) (described above).

The large number of different connexin genes that have been isolated from mice, and the co-expression of several different connexins in many cell types suggest that in addition to homo-oligomeric gap junction connexons, a large number of hetero-oligomeric gap junction connexons, composed of more than one connexin isotype exist. Evidence for the assembly of hetero-oligomeric gap junction connexons has been obtained recently by studying connexin subunit assembly in vitro, in cell-free and heterologous expression systems (Konig and Zampighi, 1995; Stauffer, 1995; Brink et al., 1997; Falk et al., 1997; Wang and Peracchia, 1998; Ahmad et al., 1999; He et al., 1999), and in vivo, in lens fiber cells (Jiang and Goodenough, 1996). By studying connexin assembly in cell-free translation/membrane protein translocation systems we also found that different connexin isotypes do not assemble in random order, but interact selectively, allowing only the assembly of homo-oligomeric and certain types of hetero-oligomeric connexons (Falk et al., 1997, 1998; reviewed in Yeager et al., 1998; Falk, 2000 a) (Figure 2).

Signals regulating connexin polypeptide assembly

The precise connexin subunit composition, stoichiometry, and organization within the connexon are likely to play a critical role in determining the properties of such hetero-oligomeric gap junction connexons. In an attempt to characterize such signals in the connexin polypeptides we have co-translated different connexin isotypes, and amino- and carboxy-terminal truncated connexins in cell-free translation systems supplemented with ER-derived membrane vesicles (microsomes), and investigated interaction and assembly of the subunit proteins by hydrodynamic and immunoprecipitation analyses. Our results are summarized in Figure 2. The results suggest that an "assembly" signal regulating principal connexin subunit recognition may be located in the C-terminal portion (preferentially third transmembrane domain) of the connexin polypeptides (Figure 2A), while a "selectivity" signal regulating specific assembly of hetero-oligomeric connexons is located in the amino-terminal portion (NH_2-terminal, first transmembrane, or first extracellular domain) of the connexin polypeptide sequence (Figure 2B, C) (Falk et al., 1997, 1998) (reviewed in (Yeager et al., 1998; Falk, 2000 a)). The high-resolution structure of gap junction channels (Unger et al., 1999) clearly demonstrates that individual connexin polypeptides within the connexon interact with one or two transmembrane domains of the adjacent connexin polypeptides. Therefore, it appears likely that specific signals regulating selective connexon assembly are located within the transmembrane domains, and/or within juxtamembrane connexin sequences.

Hypothetically, the diversity of gap junction channel subtypes is even further broadened by the possibility that connexons assembled from different connexin isotypes that are synthesized by different adjacent cell types dock into hetero-typic gap junction channels. This is suggested in vitro by the co-culture experiments of tissue culture cells that express different connexin isotypes (Elfgang et al., 1995; Valiunas et al., 2000), the pairing of *Xenopus* oocytes expressing two different connexin isotypes (Wang and Peracchia, 1998), as well as in vivo by the characterization of gap junctions between different cell types of the central nervous system (reviewed in Zahs, 1998; Nagy and Rash, 2000), or different cell types of the eye (reviewed in Vaney and Weiler, 2000). Future studies may elucidate the multiple functions that so many different gap junction channel subtypes may have.

Organization and structural composition of gap junctions revealed in live cells

The recent availability of cDNAs encoding autofluorescent proteins, such as green fluorescent protein (GFP), allows to observe the behavior of expressed proteins in live cells when their cDNA is fused with the cDNA of the protein of interest (see (Misteli and Spector, 1997) for a review). GFP, although isolated and cloned from a cold-water jellyfish (Prasher et al., 1992; Inouye and Tsuji, 1994) was genetically engineered to fold efficiently in eukaryotic cells, and to emit strong green fluorescent light upon excitation with blue light (Heim et al., 1995; Siemering et al., 1996; Kahn et al., 1997). Furthermore, GFP was found in most cases not to interfere with the normal behavior of the tagged proteins. This inert behavior was attributed to the unique tightly packed can-shape structure of GFP (Ormö et al., 1996). The availability of such a powerful

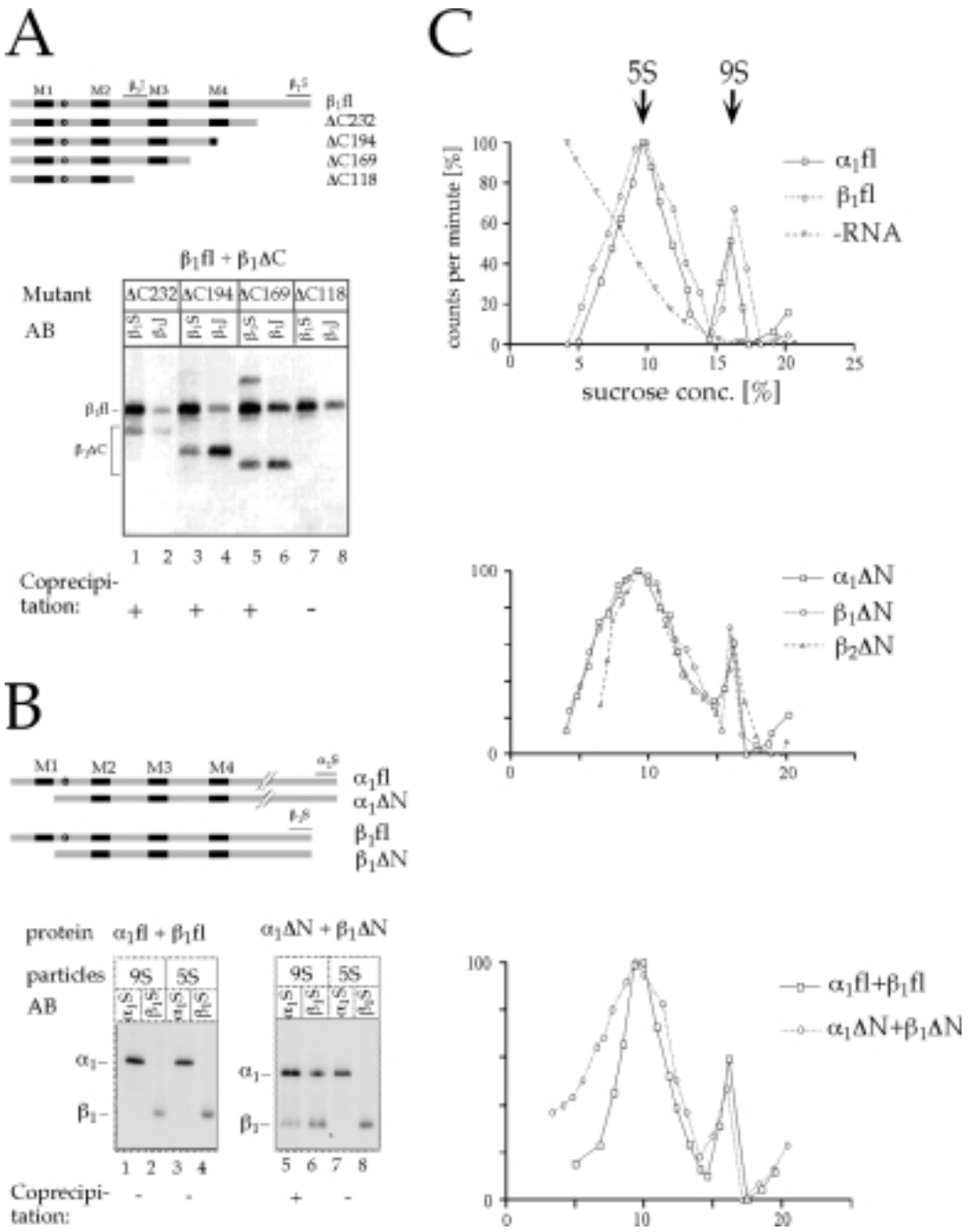

Fig. 2. Assembly of gap junction hemichannels in cell-free translation/membrane protein translocation systems, and characterization of selectivity and assembly signals within the connexin polypeptide sequences. Different connexin isotypes were translated either as full-length, or as truncated polypeptides in reticulocyte lysates in the presence of endoplasmic reticulum-derived membrane vesicles (microsomes), and assembly of the polypeptides was analyzed by immunoprecipitation (**A**, **B**), and hydrodynamic analyses (**C**). (**A**) A C-terminal portion, including the third transmembrane domain (M3), appears to be required for successful connexin subunit recognition and assembly. Full-length (fl) β_1(Cx32) was cotranslated with progressively C-terminal truncated (ΔC) β_1(Cx32) polypeptides and interaction and assembly were investigated using specific monoclonal antibodies (AB) β_1J, and β_1S. Co-precipitation of the truncated polypeptides ΔC232 to ΔC169, but not of ΔC118 was observed. A schematic representation of the polypeptides used in this analysis is shown. The transmembrane regions are marked M1 to M4. (**B**) A signal regulating assembly specificity appears to be located in the N-terminal portion, including the first transmembrane domain (M1) of the connexin polypeptides. α_1(Cx43) and β_1(Cx32) were co-translated either as full-length or as amino-terminal truncated (ΔN) polypeptides. Composition of the assembled connexons was analyzed by immunoprecipitation using specific monoclonal antibodies (α_1S, β_1S). No co-precipitation of the other connexin isotype was observed when full-length α_1(Cx43) and β_1(Cx32) were co-translated. However, coprecipitation of the other connexin isotype was observed when N-terminally truncated connexins were co-translated. A schematic representation of the polypeptides used in this analysis is shown. (**C**) Hydrodynamic analysis of connexins expressed in cell-free extracts. Assembly into connexons (9S particles) was observed in all instances. Unassembled connexins have a sedimentation coefficient of 5S. Adapted from Falk et al. (1997), and Falk (2000 a) by kind permissions of Oxford University Press and Academic Press, respectively.

reporter tool triggered the development of improved GFP variants with shifted excitation, and emission spectra (Heim et al., 1995; Heim and Tsien, 1996; Yang et al., 1998), as well as more sensitive recording devices, such as cooled charge coupled device (CCD) cameras, faster processing hard- and software that could handle large image files, and the development, improvement, and commercialization of novel imaging systems.

We have used a fluorescence enhanced GFP variant (EGFP), and its cyan (ECFP), and yellow (EYFP) color variants (Clontech, Palo Alto, CA) as autofluorescent reporters, and combined this reporter technology with single, and dual-color fluorescence, high resolution, deconvolution (DV) microscopy and computational three-dimensional volume reconstruction to study the structure, dynamics, synthesis, and degradation of gap junctions in live cells. Comprehensive biochemical, immunological, ultrastructural, and functional analysis verified that the tagged connexins trafficked normally, assembled into functional gap junction channels, and clustered into typical gap junction plaques indistinguishable from gap junction channels assembled from wild-type connexins, even under conditions where all connexin polypeptides were tagged with GFP, and no endogenous connexins were present (Figs. 3, 4) (Falk, manuscript submitted). Comparable results were obtained by Jordan et al. (1999), Holm et al. (1999), and Bukauskas et al. (2000) who investigated an α_1(Cx43)/GFP chimera.

The deconvolution process computationally reassigns the fluorescent blur, generated by out-of-focus fluorescence and lens aberration to its source using an inverse matrix algorithm to noticeably enhance the resolution of the images (see (Shaw, 1998; Falk and Lauf [manuscript submitted]) for methodological reviews). The system we used (DeltaVision Model 283, Applied Precision Inc., Issaquah, WA) allowed us to acquire images of gap junction plaques in which a single pixel of the CCD cameras photochip corresponded to 45 nm $\times$ 45 nm, or approximately 5 $\times$ 5 gap junction channels (Revel and Karnovsky, 1967) when images were taken at maximal magnification (100$\times$ objective lens plus 1.5$\times$ auxiliary magnification), although, the wavelength of the light used for imaging may reduce this theoretical resolution to a certain extent. Such a high light microscopic resolution allowed us to resolve the detailed organization of gap junction plaques in live cells (Figures 5 and 6). Similar details were seen previously only by electron microscopy. Electron microscopy, however, is only applicable in fixed or cryo-preserved cells, can only image a thin section, or a fragment of a plane, and does not allow a quantitative distinction of proteins (see related article by Benedetti et al. (2000), this issue).

High-resolution DV microscopic analysis of gap junction plaques assembled from GFP-tagged connexins in live cells revealed that gap junction plaques can be quite diverse (Figure 5). Most plaques were irregular in shape rather than circular. The edges of the plaques were found to be wavy, or jagged, and sometimes long extending protrusions, or deep invaginations deprived of gap junction channels were found to extend from their edges. Fluorescent vesicles were found at the edge and in close vicinity of the plaques, as well as further away in the cytoplasm of the cells that may represent transport vesicles, and/or partial plaque degradation products. Finally, circular, and sometimes irregularly shaped areas (approx. 0.1 – 1 μm in diameter) lacking gap junction channels (termed nonjunctional membranes in Friend and Gilula, 1972) were clearly resolved within the gap junction channel plaques (Figures 5 and 6). The characteristics described above were seen in smaller ($\leq$ 5 μm diameter), and larger ($\geq$ 5 μm diameter) gap junction plaques, in gap junctions assembled from GFP-tagged α_1(Cx43), β_1(Cx32), or β_2(Cx26), as well as in gap junction plaques assembled from wild-type connexins which were transfected or endogenously expressed and fluorescently labeled with connexin-specific antibodies (Falk; Clement et al.; Falk and Lauf, manuscripts submitted).

Furthermore, tagging connexins with autofluorescent tracer proteins in combination with deconvolution microscopy and volume rendering allowed us for the first time to evolve gap junctions, appearing as one-dimensional puncta, or lines with conventional microscopy techniques (Figures 3 and 4), into realistic three-dimensional plaques as they appear in the adjoining membranes between live cells (see Figure 5). The volume reconstructions can further be animated to even better demonstrate the true gap junction structure (Falk, manuscript submitted).

Structural composition of gap junctions assembled from more than one connexin isotype

To obtain detailed information on the structural composition of gap junction plaques assembled from two different connexin isotypes, we tagged α_1(Cx43), β_1(Cx32), and β_2(Cx26) with the GFP color variants CFP and YFP, respectively, and imaged both in cotransfected live HeLa cells using specific filter sets. Both connexins assembled into gap junction plaques that contained both connexin isotypes (Figure 6). When tagged β_1(Cx32) was cotransfected with tagged β_2(Cx26), the two proteins were distributed homogenously throughout the plaque resulting in yellow colored plaques (Figure 6, top panel). When tagged α_1(Cx43), and β_2(Cx26) were coexpressed, still both proteins trafficked to the same gap junction plaques, however, assembled into well separated domains consisting of either connexin isotype (Figure 6, center, and bottom panels). Identical results were obtained when α_1(Cx43), and β_1(Cx32) were coexpressed (shown in Falk; Falk and Lauf, manuscripts submitted). Control experiments demonstrated that the tags did not influence this specific assembly behavior (see Falk, manuscript submitted). These results suggest that coexpressed connexins sharing the same characteristics assemble into hetero-oligomeric, and probably hetero-typic channels, while coexpressed connexins with different characteristics only oligomerized into homo-oligomeric, and homo-typic channels, and only arrange into well separated domains. This hypothesis is consistent with the results obtained by investigating the homo-, and hetero-oligomeric composition of connexons, and complete gap junction channels by biochemical, immunological, and electrophysiological approaches (Elfgang et al., 1995; Stauffer, 1995; Jiang and Goodenough, 1996; Falk et al., 1997; Brink et al., 1997; He et al., 1999). A mixing of gap junction channels assembled from different connexin isotypes within the same plaque has been seen before. However, either image resolution was insufficient (Risek et al., 1994), or the imaging techniques did not allow the quantitative separation of the different channel subtypes (Risek et al., 1994; Hülser et al., 1997; Ko et al., 1999). An efficient and complete separation into either hetero-, or homo-oligomeric gap junction channel types appears convincing if subclass-specific signals, responsible for connexin subunit recognition and assembly are present in the connexin polypeptides, as our results described above suggest

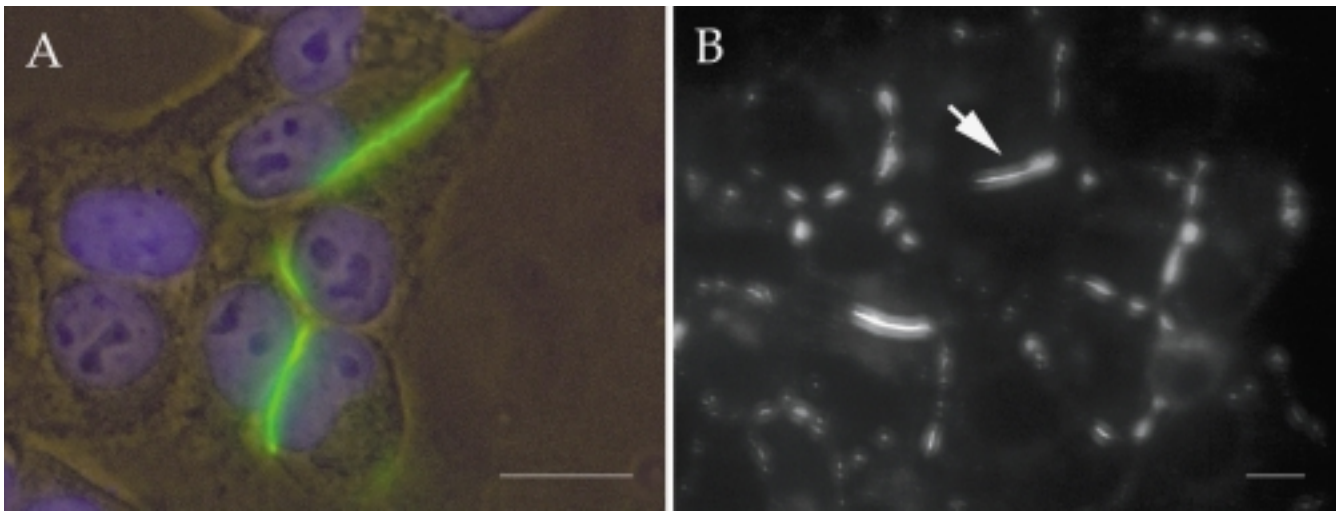

Fig. 3. GFP-tagged connexins assemble into typical gap junction plaques. Connexins α_1(Cx43), β_1(Cx32), and β_2(Cx26) tagged on their C-terminus with GFP assembled into typical gap junction plaques in the adjoining plasma membranes of neighboring transfected cells (green lines (**A**), and white lines and puncta (**B**), shown for α_1(Cx43)). In (**A**), fluorescence was imaged successively with FITC and DAPI filter sets (gap junctions in *green*, nuclei in *blue*, respectively) followed by phase contrast (cell shape). In previous publications, gap junctions were typically presented at low magnification and low image resolution as in the image shown in (**B**). We have imaged gap junction plaques, such as the one labeled with an *arrow* (**B**), at high primary magnification using deconvolution fluorescence microscopy. This technique revealed the detailed structural composition of gap junctions in live cells. Such high-resolution images and volume reconstructions of gap junctions are shown in Figures 5 and 6. Bars = 20 μm.

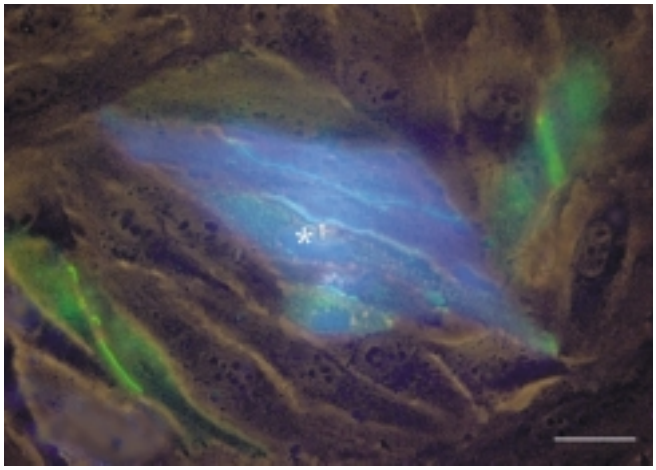

Fig. 4. GFP-tagged connexins are functional. A single cell (marked with an *asterisk*) in a cluster of HeLa cells transiently transfected with α_1(Cx43) was microinjected with a gap junction-permeable dye, cascade blue (see Falk, manuscript submitted for additional dyes). The dye quickly spread to the neighboring cells coupled via gap junctions. Gap junction plaques in dye-containing cells appear *cyan* due to the overlay of green and blue fluorescence. Gap junction plaques between non-injected cells are *green*. Blue and green fluorescence emissions and phase contrast were acquired successively in this triple exposure image. Bar = 20 μm.

(Falk et al., 1997, 1998; Falk, 2000 a). Further experiments should uncover the intrinsic characteristics of such signals.

Dynamics of gap junctions imaged in live cells

Autofluorescently tagged connexins can further be used to perform time-lapse imaging to record the assembly, degradation, and dynamic behavior of gap junctions in live cells over time. Initial studies on the dynamics of gap junctions assembled from α_1(Cx43)/GFP chimera using conventional wide-field fluorescence, and low-magnification confocal microscopy have now been published, and a lateral movement of plaques in the plane of the membrane that can result in plaque fusion and plaque disassembly, as well as some vesicle movements has been observed (Jordan et al., 1999; Holm et al., 1999). We have combined time-lapse imaging with single- and dual-color high resolution DV microscopy to record the detailed movements, as well as the assembly and degradation of gap junction plaques (M. Falk, unpublished results). The movements are complex, and at least four different types of movement could be characterized (M. Falk, manuscript in preparation). Experiments investigating the forces that drive the movements are currently carried out in the author's laboratory.

Concluding remarks

Tagging connexins with autofluorescent tracer proteins and deconvolution microscopy has allowed us to study gap junctions in living cells and to elucidate several new aspects of gap junction structure, assembly, and degradation. For the first time, entire gap junctions were imaged and reconstructed as they appear in the adjoining membranes of neighboring cells. Furthermore, a complete segregation or homogenous mixing of channels within gap junction plaques according to their characteristics was observed. Finally, time-lapse recordings have demonstrated that gap junctions are highly mobile and very dynamic structures, and that the individual channels within the plaques are turned over constantly by the fusion of newly inserted channels with the plaque, and permanent removal of channels from the plaque. This dynamic nature of gap junctions is probably an adaptation to the function of gap junctions that allows to quickly modulate their function according to modified requirements. In future experiments, it

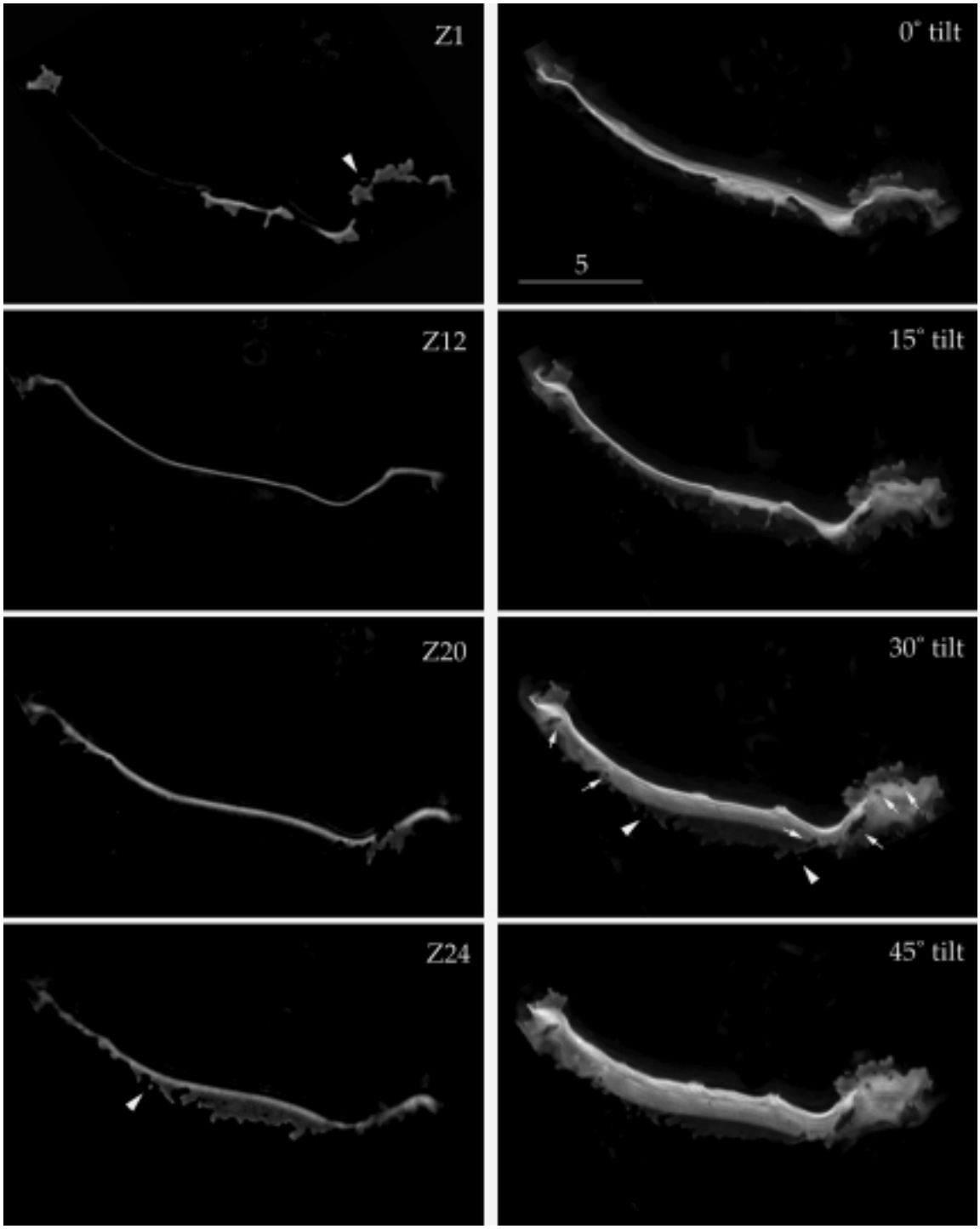

Fig. 5. Three-dimensional volume reconstruction of a gap junction channel plaque. Z-sections covering the whole depth of the plaque were acquired with 0.2 μm image spacing from live HeLa cells transfected with GFP-tagged α_1(Cx43), images were deconvolved, and volume views of the gap junction plaque were reconstructed using DeltaVision (Applied Precision Inc., Issaquah, WA, USA) *softWoRx* software. The *left panels* show selected Z-section images (Z1, Z12, Z20, and Z24). Note, that only thin sections of the plaque, as acquired with a confocal microscope, are visible on the individual images. In the *right panels* three-dimensional volume reconstructions computationally rendered from the entire stack of 40 Z-sections are shown tilted 0°, 15°, 30°, and 45° along the longitudinal axis of the plaque. Such volume reconstructions demonstrate that gap junction plaques appearing as one-dimensional lines and puncta in edge-on views indeed represent two-dimensional sheets. Furthermore, they provide a realistic, three-dimensional impression of gap junctions as they appear in the adjoining membranes between live cells. Note the structural organization of the plaques including wavy, and jagged edges, deep invaginations, long protrusions, regular and irregular-shaped nonjunctional membranes not containing gap junction channels (dark holes) within the junctional membranes (marked with *arrows* in the 30° tilt projection), as well as vesicular structures containing gap junction channels close to the edge of the plaque (marked with *arrowheads* in the images labeled Z1, Z24, and 30° tilt) (see Falk, manuscript submitted for animated volume reconstructions of gap junction plaques). Bar = 5 μm.

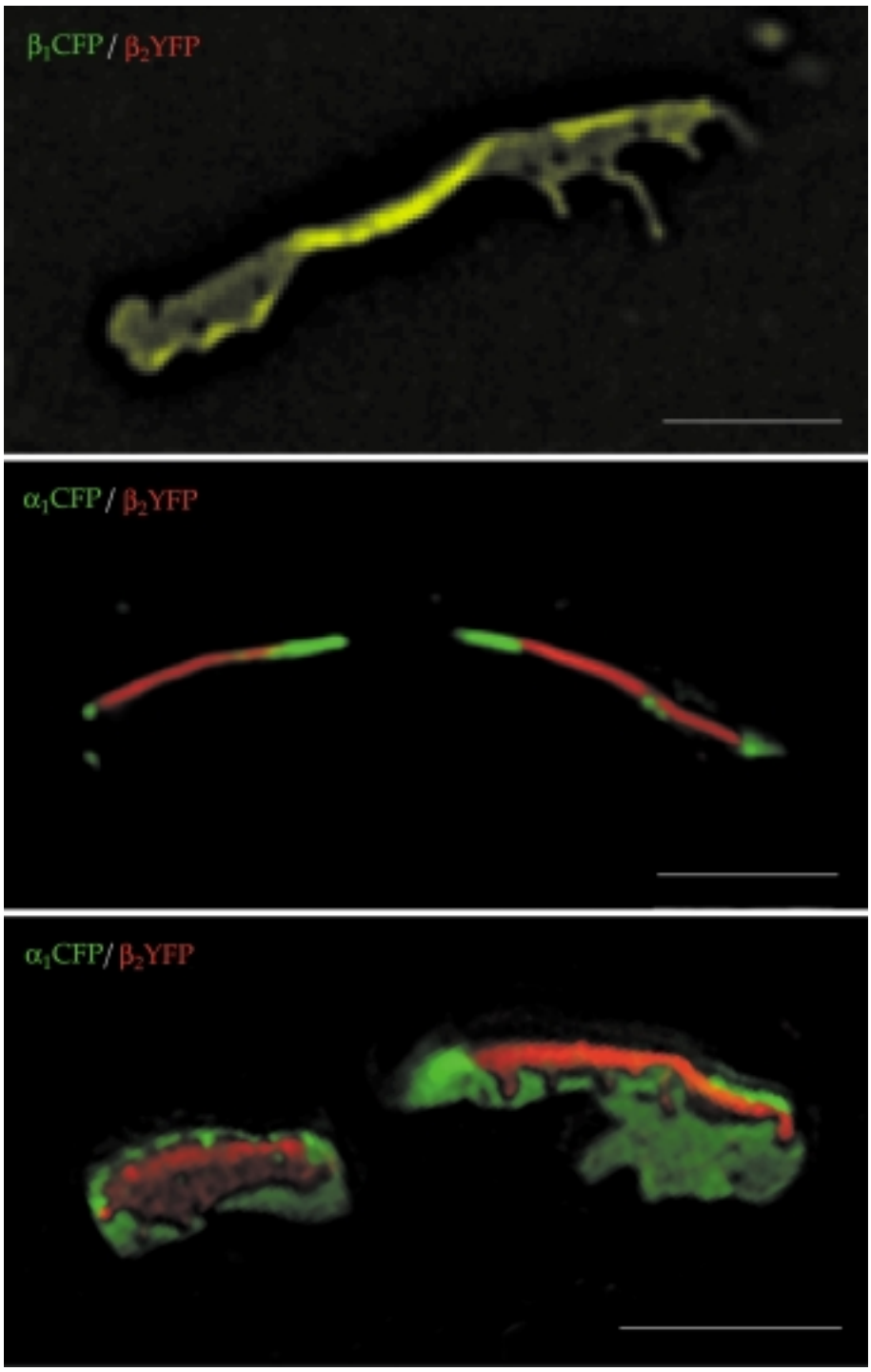

Fig. 6. Structural composition of gap junctions in live cells assembled from two different connexin isotypes. Connexin isotypes α_1(Cx43), β_1(Cx32), and β_2(Cx26) were tagged with the autofluorescent reporter proteins CFP and YFP, respectively. HeLa cells were cotransfected, and assembled plaques were imaged at high primary magnification ($100\times$ objective lens, plus $1.5\times$ auxiliary magnification). Views onto the edge, and onto the plain of the plaques are shown. In the images, CFP emission was pseudocolored *green*, and YFP emission was pseudocolored *red*. Images are shown after deconvolution. In all instances both connexin isotypes trafficked to the same junctional site. Connexin isotypes sharing the same characteristics, such as β_1(Cx32) and β_2(Cx26) probably assembled into hetero-oligomeric channels composed of both connexin isotypes (red and green, appearing homogenously yellow) (*top panel*). However, connexin isotypes with different characteristics, such as α_1(Cx43) and β_2(Cx26) assembled into well separated domains (red or green, *middle* and *bottom panels*) (see Falk; and Falk and Lauf, manuscripts submitted for images of gap junctions assembled from α_1(Cx43) and β_1(Cx32)). Bars = 5 μm.

has to be established whether channel segregation is temporary, or permanent, how the composition and dynamic nature of the plaques correlates with their function in direct cell-to-cell communication, and how precisely gap junctions are synthesized and degraded. Thus, deconvolution microscopy, fluorescence tagging, time-lapse imaging, and volume rendering further promise to be indispensable tools for investigating the biosynthesis, dynamics, and function of gap junctions.

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