Connexin Type and Fluorescent Protein-fusion Tag Determine Structural Stability of Gap Junction Plaques*

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*Running title: Gap junction plaque organization

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Background: Connexin proteins form gap junction channel clusters termed plaques. Results: Microscopy with fluorescent protein-tagged connexins showed connexin26 and connexin30 plaque arrangement is dynamic but gap junctions of connexin43 are stabilized by its C-terminal region. Conclusion: The arrangement of channels in gap junctions depends on connexin type. Significance: These findings help clarify how gap junction plaque dynamics and composition could modulate intercellular communication.

ABSTRACT

Gap junction (GJ) plaques are made up of plaques of laterally clustered intercellular channels and the membranes in which the channels are embedded. Arrangement of channels within a plaque determines subcellular distribution of connexin binding partners and sites of intercellular signaling. Here, we report the discovery that some connexin types form plaque structures with strikingly different degrees of fluidity in the arrangement of the GJ channel subcomponents of the GJ plaque. We uncovered this property of GJs by applying fluorescence recovery after photobleaching (FRAP) to GJs formed from connexins fused with fluorescent protein tags. We found that connexin (Cx) 26 (Cx26) and Cx30 GJs readily diffuse within the plaque structures while Cx43 GJs remain persistently immobile for more than 2 min after bleaching. The cytoplasmic COOH-terminus of Cx43 was required for stability of Cx43 plaque arrangement. We provide evidence that these qualitative differences in GJ arrangement stability reflect endogenous characteristics, with the caveat that the sizes of the GJs examined were necessarily large for these measurements. We also uncovered an unrecognized effect of non-monomerized fluorescent protein on the dynamically arranged GJs and the organization of plaques composed of multiple connexin-types. Together, these findings redefine our understanding of the GJ plaque structure and should be considered in future studies using fluorescent protein tags to probe dynamics of highly ordered protein complexes.
many individual GJ channels are termed gap junction plaques. The advent of green fluorescent proteins and derivatives for use as fusion proteins (1-3) allowed observation of the live characteristics of connexins within the GJ plaque structure. The results of initial pioneering studies have yielded some hard to reconcile results. For example, some live imaging studies indicated that GJs can have extremely static arrangements over the course of hours (approximating the lifetime of the proteins within the structure) while other studies indicated a large amount of rearrangement of channels within the plaques (4-8). Even in situations where very static plaque arrangement was observed, “global” plaque shape changes were observed over a short time scale (6) i.e. large sections of the plaque can split or fuse without major rearrangement of individual GJ channels relative to the position of neighboring channels. We wondered how local order is maintained during large-scale plaque morphology changes and have used new fluorescent proteins and fast live confocal microscopy to provide insights into GJ plaque structural dynamics.

Connexin subunits are assembled into hexameric connexons (half GJs) in the endoplasmic reticulum or the Golgi apparatus depending on connexin type (9-11). Upon traffic to the membrane (12), connexons diffuse in the plane of the membrane and when encountering appositional connexons can dock via interactions of extracellular loops. This docking process occurs in parallel with the clustering of the newly formed GJ channels into an orthogonal array with positioning constrained by the cellular membranes of the two junctional cells. Molecular mechanisms underlying this important cellular process are not well understood.

Almost all cells express multiple connexin genes. Connexins can come together to form mixed-type GJs, either heterotypic with connexons of one type pairing with connexons of another, or heteromeric, where connexons are heterogeneous with respect to connexin composition, see (13). Beginning with the earliest groundbreaking imaging studies of exogenously expressed connexins tagged with different wavelength-emitting fluorescent proteins it has been thought that connexins of different gene classes (alpha type such as α1 Cx43, and beta type such as β2 Cx26 or β6 Cx30) are restricted to segregated pools within the same GJ plaque (6,14-17). GJ channel characteristics and non-channel based cellular functions vary by connexin type. Posttranslational modifications to connexins can be spatially arranged into specific patterns within the GJ plaque and modulate intercellular communication (18,19).

GJ plaque arrangement stability and steady-state structure are both potentially important to cellular function and intercellular communication. Therefore, we reexamined these issues. We focus on connexins co-expressed in mammalian astrocytes using new generation fluorescent protein tags optimized with respect to correct folding and lack of dimerization. Imprecise terminology for FRAP studies of GJ biology has led to confusion when comparing results from different studies. The persistence of GJ plaques at the membrane (often referred to as gap junction stability), and the movement of whole plaque structures in the membrane (plaque mobility) describe macro-behavior of GJs. We examine the stability of the arrangement of the GJ channels within the GJ plaque (plaque arrangement stability) of astrocyte-expressed connexins Cx43, Cx30, and Cx26.

EXPERIMENTAL PROCEDURES

Cell culture and transfection- HeLa cells were plated into 8 well imaging chambers (ibidi, LCC) and each well transfected with 0.5 μg of each plasmid to drive expression of Connexin-fluorescent protein fusions 24-72 h prior to imaging. Optifect (Life Technologies) was used to transfect the plasmids according to manufacturer’s instructions adjusted for the surface area of the wells of the ibidi chambers (50 μl of Optimem media and 3 μl of Optifect reagent per well). Optimem media was replaced with the standard growth media for HeLa and N2A cells (DMEM with 10% fetal bovine serum and 1% Penicillin-Streptomycin), or the standard growth media of RINm cells (RPMI1640 media with 10% fetal bovine serum and 1% Penicillin-Streptomycin) 12-16 h after transfection. Cells were incubated in standard growth media for at least 6 h prior to imaging.

Plasmids and subcloning- The coding sequence (CDS) for Cx43, Cx30, and Cx26, were inserted in-frame into plasmids based on the Clontech EGFP-N1 and EGFP-C1 backbone.
Plasmids used for this study contain the CMV promoter with a short linker between a N or C terminal superfolder (sf) GFP, enhanced blue fluorescent protein 2 (EBFP2) or monomerized Venus (mVenus) tags (20,21). Monomeric-sfGFP (msfGFP) contains a V206K monomerizing mutation (22-24). Superfolder GFP (sfGFP),(25) contains a valine residue encoded at position 206 in the fluorescent protein sequence and GFP derivatives (e.g. EGFP, ECFP, and EYFP) with an alanine or valine at this position have a weak tendency to dimerize (22-24). Cx26 and Cx43 coding sequences used for fluorescent protein fusion constructs were from rat. All Cx30 constructs were made with the human GJB6 CDS. Untagged Cx26 and Cx43 expression constructs for immunostaining were from mouse Gjb2 and Gja1 CDS respectively. Rodent and human connexin protein sequences are highly homologous within connexin type (e.g. human and rat Cx43 and Cx30 protein sequences are 97% and 95% identical, respectively). The linker sequence for N1 plasmids (those constructs with the FP tag on the COOH-terminus (CT) of the connexin) containing rat Cx26 is ADPPVAT, YPDPPVAT for human Cx30, and for rat Cx43 is SADPPVAT. The linker is SGLRSRAQ for all C1 plasmids (constructs with the FP tag on the amino-terminus of the connexin). Plasmids were sequenced from both ends of the coding region for overlapping sequence verification. The Cx43K258stop truncation was made using PCR cloning with the InFusionHD kit: Rat Cx43 with a stop codon inserted at the 258th position (and the rest of the CT omitted) was inserted into the multiple cloning site of sfGFP-C1 and EBFP2-C1 plasmids. The Cx43K258stop sequence was transferred to sfGFP-N1 and msfGFP-N1 plasmids, in frame with the fluorescent protein and with the stop codon removed. This plasmid construction was performed with the InFusionHD PCR cloning kit. The truncation constructs used the same linker sequence as the full-length Cx43 constructs. Plasmids used for this study are available at the online repository Addgene.com.

**Microscopy and FRAP**- Fifteen to 30 min prior to imaging, media was removed and replaced with imaging media (26) containing 10% fetal bovine serum, 25 mM HEPES and 2 mM glutamine added, in DMEM without phenol-red. The ibidi imaging chambers were then placed in the incubator for 10 to 15 min before transport to the confocal microscope where they were kept in a chamber maintained at 37°C on the stage of a Zeiss LSM 510 Live with Duo module and imaged with a 63X NA=1.4 oil immersion objective. The detector consists of dual 512 pixel linear arrays of CCD camera-type pixels. Pixel size was 200 nm for FRAP experiments and 100 nm for 3D Z-stacks. Cells were imaged at 500 ms intervals with 405 nm (EBFP2), 489 nm (GFP variants, mVenus, Alexa Fluor 488), and 561 nm (Alexa Fluor 594) laser illumination for both image acquisition and bleaching. For general FRAP experiments, the bleach region was set to bleach 2 µm of the GJ plaque aligned on end, which in single Z-plane time-lapse acquisitions forms a line that is in practical terms, limited to one dimension by the resolution limit of standard light microscopy. Bleaching for FRAP with an 80/20 splitter in place and laser power set to 100% was performed on membrane regions 2x4 µm rectangle at scan speed of 5 frames/s and 3 iterations for GFP and 5 iterations for EBFP2. A serial Z-section through the cells was conducted before each FRAP experiment. Each FRAP experiment consisted of 20 pre-bleach acquisitions followed by bleaching the region of interest (bleach-ROI) which was followed by 220 single plane scans at 500 ms intervals. Immediately following the final single-plane acquisition of the FRAP experiment a second z-section was performed as close to 2 min after the bleach time-point as possible. Due to the high sensitivity of microscope detectors and high signal intensity from the sfGFP fluorophores on each GJ (12 per GJ channel), the laser power required for post-bleach time-lapse acquisitions was exceptionally low, resulting in minimal acquisition photobleaching.

**Heteromeric gap junction FRAP**- HeLa and N2A cells were co-transfected with plasmids. For heteromeric GJ experiments with untagged connexins the tagged and untagged connexin plasmids were premixed at a ratio of 1:4 with 500 ng of the plasmid encoding fluorescent protein tagged connexin and 2 µg of the plasmid encoding the untagged plasmid. The same imaging and bleaching settings were used for heteromeric FRAP as was used for all-tagged-connexin FRAP experiments except an additional higher intensity Z-stack was acquired before and after the FRAP experiment since the heteromeric plaques were
variable in intensity but generally at least 50% dimmer than GJs made up of purely tagged connexins.

**Heterotypic gap junction FRAP** Cells were transfected in separate culture dishes with different plasmids. 12 h after transfection the cells were washed once in growth media, twice in PBS, and then one of the dishes of cells was trypsinized and replated into the container of the other cells with the other transfection. One group of cells was transfected with sfGFP tagged connexin in the wells of the 8well ibidi chambered slide, each well of the slide received 1 µg of DNA. The other group was transfected in a 3.5cm culture dish with 4 µg of DNA for plasmids containing un-tagged Cx43 (CMV promoter driven mouse Cx26 and EF1α promoter driven rat Cx43 and rat Cx43S257stop). Cells sourced from the dish transfected with untagged connexin were marked in the following ways: N2A cells were loaded with CellTracker orange CMTMR (Thermo Fisher, Cat. No. C2927) 12 h after the transfection and prior to replating into the ibidi 8-well chambered slide. The HeLa cells were from a line stably expressing CMV-promoter driven mCherry (cytoplasmic localized). The resulting cultures contained both homotypic GJs (tagged::tagged, untagged::untagged) and heterotypic GJs (tagged::untagged). The heterotypic GJs were identified first by their lower fluorescence and shape of Cx-msfGFP localization (half of the cell-pair without expression). The heterotypic GJs were then confirmed by the presence of orange dye (N2A cells) or mCherry (HeLa cells) in one of the two cells- the cell not expressing Cx-msfGFP. FRAP was performed with the same imaging and bleaching settings as was used for other FRAP experiments.

**Immunostaining** HeLa cells grown in 8-well ibidi dishes were transfected 48 h prior to immunostaining with combinations of Cx43, Cx26, Cx26-msfGFP, msfGFP-Cx26, sfGFP-Cx26 and/or Cx26-sfGFP. 12 h post- transfection mixture (Optifect+DNA+Optimem media), the Optimem media was replaced with DMEM with 10%FBS and Pen/Strep. Cells were allowed to grow for 24 h then were washed two times with PBS then fixed for 20 min in 4% paraformaldehyde in PBS at RT. Cells were washed three times in PBS then incubated in blocking solution (4% Goat Serum and 0.1% Triton-X 100 in PBS) for 1 h. Cells were then washed twice with PBS, then primary antibody was applied. Cells expressing untagged Cx43 were stained with rabbit polyclonal antibody against Cx43 CT, 1:500 (C6219, Sigma) for 16 h at 4°C. Cells expressing untagged Cx43 and Cx26 were stained with mouse anti-Cx26 antibody (30-8100, Invitrogen) in addition to the same Cx43 antibody and in the same conditions as above. Cells were then washed six times in PBS and then stained with Alexa Fluor594 labeled donkey anti-rabbit secondary antibody (1:1000) for 1 hr at RT (Alexa Fluor488 labeled goat anti-mouse secondary antibody was included for staining Cx43 and Cx26). Cells were then washed 6 times in PBS and imaged using the Zeiss 5Live microscope with a 63x PlanApo 1.4NA oil objective.

**FRAP analysis** To generate recovery curves, the bleach region was outlined as the region of interest (ROI) and the fluorescent protein pool was defined as the entire plaque within the focal plane (Fig. 1). Recovery curves were generated by correcting for loss of signal due to bleach and for acquisition-bleach of the total pool of fluorescent protein and normalized to 100% pre-bleach and 0% for the initial post-bleach time-point to normalize for incomplete bleaching within the bleach ROI as previously described (27). Background values were subtracted from both the bleach ROI and the fluorescence pool ROI. A correction factor (cf) was calculated by dividing the average of the 10 fluorescence pool readings preceding the bleach (initial fluorescence of the fluorescence pool; fpFo) by the fluorescent pool ROI readings at each time point (Fp), (fpFo/Fp). The bleach ROI reading (bf) for each time point was divided by the bleach region baseline-initial fluorescence Fo (bf0), (bf/bf0) and the resulting fraction of initial fluorescence was then multiplied by the correction factor. The resulting corrected fractional fluorescence was then multiplied by 100% to calculate “normalized recovery (%).” With omission of background subtraction, transforming to complete bleach baseline, and averaging to generate initial fluorescence pool values, the calculations for the recovery curve values were as follows: Normalized percent FRAP values at each time point were calculated with the following equation, FRAP%=(fpFo/Fp)*(bf/bf0)*100%
Correction for loss of signal due to bleach was necessary since the 2 µm linear bleach area eliminated a substantial portion of the total fluorescence pool (average 43±15%, 38±15, 48±14 for Cx43, Cx30, and Cx26 –msfGFP, respectively shown in Fig. 1). The 2 µm linear bleach size was chosen because it was small enough to minimize the percent of the plaque that was bleached while large enough to help minimize the effect of cell and plaque movement which can cause significant systematic error in FRAP experiments performed on GJ plaques. The normalized data points at 30 s after the bleach time-point were used in comparison of percent recovery at 30 s.

Comparison of post-FRAP 3D reconstructions with FRAP recovery curves and percent recovery indicated that these measures do not provide absolutely reliable quantitation of stability of the plaque structure due to particular aspects of the GJ plaque such as shape changes and whole-plaque movement. Therefore, we performed an alternative analysis to minimize errors generated by variable plaque size, out of focus bleaching, and whole-plaque movement. We refer to our approach in this study as “Bleach border analysis” to differentiate it from other methods to assess protein dynamics using FRAP. Bleach border analysis examines the rate at which the sharp border between bleached and unbleached regions of the plaque (created by photobleaching of an ROI within the plaque) transitions to a diffuse blurred gradient due to rearrangement of plaque components (Fig. 2). The sharpest point in the border between bleached and unbleached sections of the plaque was identified by the peak of the absolute value of the differential of a line scan along the plaque. A border bleach value is calculated at immediately postbleach (0 s) and at 30 s post-photobleach as described in Fig. 2 legend. The ratio of the border sharpness at the two time points (0 s post-bleach peak border)/(30 s post-bleach peak border) is calculated for each FRAP experiment and then normalized to the (0 s post-bleach peak border) and expressed as a percent.

The procedure for calculation the bleach border analysis is as follows: FIJI/ImageJ software (28,29) was used to rotate the plaque to a horizontal position, then the average intensity of each pixel for 5 horizontally stacked 20 pixel-wide line scans were extracted for the first post-bleach image and the first image 30 s after the photobleach event as shown in Fig. 2 A,D. The line-scan intensity data was transferred to IGOR Pro ver. 6.36 (Wavemetrics) software where it was smoothed 3X by nearest neighbor smoothing. Example smoothed line scan plots are shown in Fig. 2 B,E. The absolute value of the differential of the smoothed curve at each data point was calculated as plotted in Fig. 2 C,F. The IGOR Pro package “Multipeak Fitting 2” was used to locate the peak value of the differential and that peak value at 0 s post-bleach and 30 s post-bleach was compared between groups. This procedure provides a measure of the sharpness of the bright-to-dark bleach-generated border and allows for some plaque movement in the x and y directions of the image sample since locating the peak of the change from light to dark effectively localizes the center of the bleach border, even if the plaque and the bleach border within it move slightly. Bleach border analysis does not correct for plaque movement in the axis of the light path, however.

Comparison of average plaque intensity and FRAP for heterotypic and heteromeric gap junctions- Intensity data for 6 of the brightest pixels (assessed visually) within the plaque from the single plane acquisition of the FRAP experiment in the time point prior to photobleaching were exported using ROI manager-measure function in ImageJ/FIJI software. The measurements were exported to Microsoft Excel and the 6 points were averaged. The per-plaque average intensity was then compared by averaging by group for heterotypic (tagged::untagged) GJ analysis. Students two-tailed T-tests were used to test for significant differences between tagged::untagged (heterotypically tagged) and tagged::tagged (homotypically tagged) plaque brightness with p<0.05 considered a significant difference. Individual plaque-average intensity was compared to the normalized percent recovery at 30 s post-bleach for heteromeric (tagged/untagged) GJ plaques. Pearson correlation was used to test if there was correlation between heteromeric plaque brightness and percent recovery at 30 s post-bleach.

Comparison of gap junction intra-plaque mixing and 3D reconstructions- A 3D reconstruction was generated as a maximum
projection reconstruction in Imaris software (Bitplane). Serial Z-sections were spaced 0.44 µm or 0.25 µm apart. An orthoslicer channel was applied to the 3D reconstruction in Imaris software and aligned by hand before export as a tiff file. The striping or moiré pattern that is present in 3D reconstructions is an artifact of the 3D reconstruction processing in conjunction with the thickness of the plaque in one dimension in comparison with the other two spatial directions (see Supplemental Movie 1). Image cropping and size adjustments were performed in Adobe Illustrator CS4 software. To measure the average length of mixed GJ plaques the images were examined as 3D maximum projection reconstructions in Zen2.1 Lite Black edition (Carl Zeiss, Inc.) to visually assess if plaques with both connexins present were intermingling or segregated by channel type. GJs were considered to be non-mixed if both connexin types were observed at the continuous structure and if there were areas within the structure, which had sub-regions that favored one color channel over the other. To measure average plaque size, the same image Z-stack was loaded into the FIJI (29) ImageJ1.48q (28). A line was drawn across the length of the GJ plaque and the measure function was used to measure the length of the each plaque in the XY plane at which the plaque was widest.

Production of supplemental movies- All supplemental movies were prepared in Zen Lite (Ver. 2012 black edition; Carl Zeiss Inc.) and/or Imaris (Bitplane) software then assembled, cropped, and captioned in Corel Video Studio Pro X4 (Corel Corporation). Images were acquired on a Zeiss 5Live Duoscan microscope with a 63x PlanApoFluor 1.4NA oil immersion objective. Reconstructions from 3D Z-stack acquisitions for supplemental movies S1-S4 were generated in ZenLite 2012 software set to reconstruct with maximum precision and no thresholding. Reconstructions were generated by rotation around the Y-axis at 5 degree steps. Example FRAP experiments in Movies S1-S4 are shown at 10X speed and re-encoded at 30 fps and assembled with 3D reconstructions using Corel Video Studio Pro X4 (FRAP movies remain at 10X speed, 120 s in real experimental-time is shown and duration of FRAP experiment as displayed in movies is 12 s). 3D FRAP movies were generated from 3D Z-stack time lapse acquisitions. Image interval is listed within the movie (between 3-5 s interval). These movies were re-encoded at 30 fps and captioned in Corel Video Studio Pro X4. Dual-color 3D reconstructions for Movies S7-S11 were generated using Imaris software as in Figs. 8-10 with key-frame manual rotation and on/off switching of individual color channels to show GJ plaque structure. Movies S7-S11 were re-encoded at 30 fps in Corel Video Studio Pro X4.

Statistics- Comparisons between data groups were performed as stated in each figure legend and as relevant in the text using GraphPad Prism ver. 6. p<0.05 was considered a significant difference between groups. Groups were compared using one way ANOVA followed by Tukey’s multiple comparisons unless otherwise noted.

RESULTS

Mobility of gap junction channels within plaques- We used fluorescence recovery after photobleach (FRAP) of monomerized-superfolder green fluorescent protein (msfGFP) tagged connexins (msfGFP attached to the CT of the connexin protein by a short linker) to test the mobility of GJ channels. We did not compare diffusion of single proteins or multimers in relation to the cell or the coverslip. Instead, we used FRAP to test for rearrangement of proteins within GJ plaques. Movement of the entire plaque structure and cell shape changes can occlude differences in intra-plaque arrangement stability. Additionally the highly variable size and geometric arrangement of GJ plaques makes standard methods of assessing diffusion coefficient and mobile fraction through analysis of single Z-plane time lapse FRAP experiments infeasible for this work (30). GJ plaques are very anisotropic structures that can vary in size from less than 100 nm to 20 µm. Our results and those of others (4-8) indicate that the majority of recovery after photobleach within a GJ plaque comes only from channels making up the plaque at the bleach time-point. This means that the fluorescence-pool available for recovery is only the GJs making up the plaque, when considered on a time-scale of less than five min. We performed FRAP experiments on plaques ranging from 4-30 µm for this study, but since our bleach size was 2 µm this led to bleaching of ~7-50% of the total fluorescence pool and up to 80% of the fluorescence-pool when only the part of the
plaque visible within the single confocal plane is considered (Fig. 1A). These challenges to analysis of mobile fraction and calculation of effective diffusion coefficient become more severe due to systematic differences in plaque size and geometry for the groups to be compared (Cx43, Cx30 and Cx26). Since gap junction plaques vary in size the percentage of fluorescence pool eliminated with a constant 2 µm bleach size was variable- leading to substantial variability in apparent percent recovery (Fig. 1A). Movement of the entire gap junction plaque within the cell led to variability in the apparent fluorescence pool as detected in single-plane confocal acquisitions over time during the post-bleach acquisition period (Fig. 1B). Together, these sources of variation led to increased error bars in measures of FRAP. Moreover, 3D curved and anisotropic geometry of plaques requires custom FRAP analysis techniques.

Given these complicating issues for the standard use of the FRAP technique, we focused our analysis on identifying factors that control the rate of rearrangement of the GJ channels that make up the plaque structure. Therefore, we compare three parameters of how rapidly the GJ channels change their position within the plaque (plaque arrangement instability). 1) For cursory comparison of plaque arrangement stability we present the recovery of average fluorescence signal within the bleached region over time and compare percent recovery at a single, early time point (30 s post bleach). 2) Since plaque size and geometry vary widely from cell to cell within the same treatment group, we also compare rearrangement with a more focused assay: we compared the rate of blurring of the bleach border (a bleached/unbleached border forms when GFP on a subsection of the plaque is photobleached). 3) We performed 3D reconstructions 2 min after photobleaching in order to avoid the effects of whole-plaque movement or cell shape change.

Cx26 and Cx30 are mobile, but Cx43 is not. We found that Cx26 (Cx26-msGFP) and Cx30 (Cx30-msGFP) GJs rapidly rearrange within the plaque while Cx43 (Cx43-msGFP) GJs did not show detectable positional rearrangement during two min of post-bleach observation in any part of the plaque structure (Fig. 3, Supplemental Movies 2-6). No detectable rearrangement (blurring of the bleach border) was observed for Cx43 in longer-term observation (up to 1 h, Fig. 3D). This behavior was reflected in much greater recovery of fluorescence for Cx26-msGFP and Cx30-msGFP in the photobleached region over the course of 2 min than for Cx43-msGFP as shown in time-lapse imaging and by normalized FRAP curves for average intensity for the bleach region over the course of 60 s (Fig. 3 A-E). The percent normalized FRAP at 30 s was significantly higher for Cx26-msGFP and Cx30-msGFP (Fig. 3F). A small but non-zero recovery for Cx43-msGFP was obtained when FRAP was analyzed as normalized percent recovery at 30s. This differed from the total absence of recovery seen with analysis of bleach-border blurring (Fig. 3F) or 3D reconstructions of the bleached plaque 2 min after photobleach (Fig. 3D, and Supplemental Movies 2 and 4). We observed that a very sharp border was created by the photobleaching event on Cx43-msGFP plaques, and it was maintained for at least two min thereafter. Rearrangement of the photobleached Cx43 was never observed in 3D reconstructions acquired up to 1 h post-bleach (Fig. 3D). Given these results, we conclude that there is no rearrangement of Cx43 channels within the plaque in our experimental conditions, and that the small apparent recovery in normalized average fluorescence of the bleach ROI was the result of plaque movement during the imaging experiment. These results are in agreement with previous reports indicating that a sharp border between bleached and unbleached tags marking Cx43 location can be maintained > 1 h after photobleach (6). These findings highlight the extreme difference in mobility behavior between Cx43 compared to Cx30 and Cx26. A difference in mobility between Cx43 and Cx26 was previously reported (5), but our new data indicate the difference is substantial. We replicated these findings (Fig. 3E) in connexin-deficient N2A (transformed neural-origin mouse cells) and RINm cells (Table 3) in order to rule out a cell-line specific effect and since HeLa cells sometimes express a very small number of Cx45 channels endogenously, resulting in channel activity detectible only by dual-cell patch clamp electrophysiological recordings (31). These core results are summarized in Table 1. The striking and reproducible differences in plaque arrangement stability that we found led us to further characterize the arrangement of GJs within...
the plaque and to identify mechanisms underlying such differences.

**Effects of fluorescent protein tags on mobility** - Previous studies on GJ mobility, trafficking and plaque structure have used a variety of fluorescent proteins as new generation tags were developed. Oligomerization of fluorescent protein tags has long been a concern (32) and sensitive new methods indicate that superfolderGFP shows an increased tendency to aggregate/oligomerize compared to superfolderGFP with a V206K monomerizing mutation (msfGFP)(22,33). When we tested for an effect of sfGFP on plaque arrangement stability, we found that Cx30 and Cx26 tagged with sfGFP had different mobility characteristics than connexins tagged with msfGFP (Fig. 4). The connexin and linker sequences were identical with the only difference being in residue 206 of the fluorescent protein tag. Rearrangement of Cx26-sfGFP and Cx30-sfGFP was significantly less than that of Cx26-msfGFP and Cx30-msfGFP at 30 s post-bleach as analyzed by comparison of percent normalized recovery (Fig. 4A-C) and by border analysis at 30 s post-bleach (Fig. 4D).

The NH2-terminus of connexin proteins is expected to be tucked into the pore of the channel based on x-ray crystallography on Cx26 and other studies (34,35). Attachment of a fluorescent protein fusion tag to the NH2-terminus (NT) of connexins has been found to eliminate channel activity but the plaques formed by the NT-tagged connexins appear similar to those formed by CT-tagged connexins and to those observed in immunostaining for un-tagged connexins over-expressed in HeLa cells (4). Relocation of the fluorescent protein tag would be expected to reposition the tag from the lateral edge of the cytoplasmic part of the GJ channels to the medial part of the channels as illustrated in Fig. 5A and B. Furthermore, a previous report in which Simek and colleagues used a different form of FRAP analysis (4) the authors noted an increased percentage of plaques with >15% recovery (the fraction of mobile protein was used as a measure of total % recovery in the Simek et al. study) when the CT of Cx43 was removed by truncation at amino acid 244(4). It is important to note that these experiments were performed with Cx43 truncated at amino acid 244, but with a non-monomerized CT GFP tag on the connexin. We performed FRAP experiments with Cx43 that we mutated to remove most of the CT by truncation. Cx43K258stop-msfGFP and sfGFP-Cx43K258stop formed very fluidly arranged plaques (Fig. 6). Cx43K258stop is listed as Cx43t258 in figures for brevity. We found that sfGFP-Cx43 truncated at amino acid 244 also formed fluidly arranged plaques with an average recovery of 23± 3% and percent “bleach border blur” of 53±3% at 30 s in 7 experiments in HeLa cells. These results indicated that the CT of Cx43 stabilizes the arrangement of channels within GJs (Fig. 6). Similar results were obtained in other connexin-deficient cell lines including N2A cells as shown in Fig. 6. Non-monomerized sfGFP tagged to the CT of Cx43K258stop had a similar stabilizing effect to that observed for Cx26 and Cx26 were more mobile than Cx26-sfGFP. Together, these results further support the hypothesis that stability observed for Cx26-sfGFP is due to interactions between fluorescent protein tags, and are summarized in Table 2. The significantly higher mobility for sfGFP-Cx26 compared to Cx26-sfGFP (Fig. 5C-F) indicates that the placement of the fluorescent protein tag at the NT of the connexin protein can occlude the effect of fluorescent protein tag aggregation on connexin mobility. However, there was no difference in plaque arrangement stability between Cx43-msfGFP (Fig. 3) and msfGFP-Cx43, sfGFP-Cx43, EBFP2-Cx43, and Cx43-mVenus (Fig. 5G, and as assessed by 3D reconstruction of Z-stacks after FRAP). These results indicate that some aspect of the Cx43 protein (other than aggregation of the fluorescent protein tag) is responsible for the observed stability of GJ arrangement for this connexin.

**The COOH-terminus of Cx43 is required for plaque arrangement stability** - The CT of Cx43 contains binding sites for many proteins that could act as a scaffold to stabilize the GJ plaque. A previous study using FRAP and non-monomerized GFP tags by another group noted a modest increase in the percentage of plaques with >15% recovery (the fraction of mobile protein was used as a measure of total % recovery in the Simek et al. study) when the CT of Cx43 was removed by truncation at amino acid 244(4). It is important to note that these experiments were performed with Cx43 truncated at amino acid 244, but with a non-monomerized CT GFP tag on the connexin. We performed FRAP experiments with Cx43 that we mutated to remove most of the CT by truncation. Cx43K258stop-msfGFP and sfGFP-Cx43K258stop formed very fluidly arranged plaques (Fig. 6). Cx43K258stop is listed as Cx43t258 in figures for brevity. We found that sfGFP-Cx43 truncated at amino acid 244 also formed fluidly arranged plaques with an average recovery of 23± 3% and percent “bleach border blur” of 53±3% at 30 s in 7 experiments in HeLa cells. These results indicated that the CT of Cx43 stabilizes the arrangement of channels within GJs (Fig. 6). Similar results were obtained in other connexin-deficient cell lines including N2A cells as shown in Fig. 6. Non-monomerized sfGFP tagged to the CT of Cx43K258stop had a similar stabilizing effect to that observed for Cx26 and
Cx30 and this effect was lost when the non-monomerized sfGFP was transferred to the NT of Cx43K258stop (Fig. 6E), further supporting separate mechanisms of stabilization for endogenous Cx43 and “artificial-stabilization” by non-monomerized fluorescent protein tags; results are summarized in Table 2.

**Cx43 stabilization of gap junction channel arrangement does not depend on fluorescent protein tag**- We found that tagged or untagged Cx43K258stop formed plaques and paired with full-length Cx43 regardless of if the full-length or truncated Cx43 was tagged (Fig. 7). This allowed us to test if un-tagged Cx43 stabilized the normally fluidly arranged Cx43K258stop-msfGFP and we found that such heterotypic Cx43::Cx43K258stop-msfGFP GJs were made up of stable plaques (Fig. 7). Untagged-Cx43S257stop::Cx43K258stop-msfGFP formed fluidly arranged plaques (not shown). Likewise, when Cx43K258stop-msfGFP was co-expressed with full length tagged (EBFP2-Cx43, not shown) or untagged Cx43 the resulting plaques were stably arranged (Fig. 7E). These results further indicate that msfGFP is not the mechanism behind Cx43 plaque arrangement stability. Co-expression of Cx43K258stop-msfGFP within the same cells as Cx43S257stop produced heteromeric plaques that were fluidly arranged indicating that the msfGFP tag did not act as a lubricating agent for the channels within the plaque (Fig. 8). We found that heteromeric plaques made up of co-expressed Cx43-msfGFP with Cx43 (full-length, untagged) or with Cx43S257stop formed stably arranged plaques (Fig. 8).

**The fluorescent protein tags do not lubricate Cx26 channel arrangement**- We found that Cx26-msfGFP::Cx26(no-tag) heterotypic GJs form fluidly arranged GJ plaques (Fig. 7E-G). Cx26-msfGFP/Cx26 heteromeric GJs have similar arrangement dynamics as GJs purely composed of Cx26-msfGFP (Fig. 8A,B). This indicates that space occupied by the fluorescent protein tag is not the cause of the fluid arrangement of Cx26 GJs.

**Fluorescent protein tag strongly affects heterotypic plaque arrangement**- Because we observed strong effects of fluorescent protein tag type and location on the stability of GJ arrangement within the plaque, we went on to test if fluorescent protein tags also affect the arrangement of the plaque when multiple connexin types were present within the same plaque. For these tests we used enhanced blue fluorescent protein tagged connexins co-transfected with msfGFP or sfGFP tagged connexins. Previous reports using non-monomerized, CT fluorescent protein tags indicated that Cx26 and Cx43 are maintained within spatially separated pools of GJs at the same plaque structure (6). When EBFP2-Cx43 was co-transfected with Cx26-sfGFP or with Cx30-sfGFP the resulting plaques formed segregated structures as shown in Fig. 9A,B. These observations are in agreement with previous reports and the current concept of GJ arrangement at the plaque as reviewed by Koval and colleagues (13). However, when we co-transfected EBFP2-Cx43 with Cx26-msfGFP (or with Cx26-mVenus, not shown) or with Cx30-msfGFP we observed mixed plaques (Fig. 9C,D). In parallel with decreasing plaque arrangement stability, translocation of sfGFP from the CT to the NT of Cx26 and Cx30 converted the plaque to the intermingled morphology when co-expressed with Cx43 (Fig. 9E,F). Even in combinations in which connexins formed intermingled plaques, we observed well separated intracellular punctate structures- likely representing distinct pools of nascent Cx26/Cx30 in separate vesicles than Cx43 en route to the plasma membrane, indicating Cx43 subunits do not form heteromeric GJs even in intermingled plaques- in agreement with previous reports (9,36,37) and illustrated in Supplemental Movie 8. Immunostaining of untagged Cx43 co-expressed with Cx26-msfGFP and Cx26-sfGFP yielded similar results as with tagged Cx43 (Fig. 10A,B). When cells expressing untagged Cx43 and Cx26 were immunostained, intermingled plaques were observed (Fig. 10A-D) with some clustering of untagged connexins observed in some other cases (see Table 4). When EBFP2-Cx26 or EBFP2-Cx30 were co-expressed with Cx43-sfGFP segregated plaques formed (Fig. 10E), but when EBFP2-tagged beta class connexins Cx30 and Cx26 were co-expressed with Cx43-msfGFP intermingled plaques formed (Fig. 10F), but very small clusters of Cx43-msfGFP were observed. Interestingly, in this case the large Cx43-sfGFP clusters localized to the center of the plaques in contrast to when EBFP2-Cx43 is co-expressed with Cx30-sfGFP, further indicating that dimerization of the sfGFP is the mechanism...
behind formation of large clusters; results are summarized in Table 4. When EBFP2-Cx26 and Cx30-sfGFP were co-transfected they formed intermingled fluorescence both at the plaque structure and in intracellular puncta, likely due to heteromeric channel formation (not shown), as previously described (6), however demonstration of heteromeric or homomeric connexin formation has not been accomplished through optical microscopy but has been demonstrated previously through biochemical and electrophysiological methods (36,37). Gong et al. raised the possibility that un-tagged Cx43 and EGFP-Cx32 might form intermingled plaques in an earlier study (38). In summary, consistent arrangement of GJs into large well defined clusters- according to class- at the same plaque structure is caused by attachment of a non-monomerized fluorescent protein tag on the CT of one of the two connexins, at least in the case of Cx43, Cx30, and Cx26.

DISCUSSION

We show that multiple factors affect the stability of GJ plaques. Our demonstration of dynamic Cx26, Cx30, and Cx43K258stop plaque arrangements establishes that plaque arrangement stability is not required for formation of large plaques. This finding is important for models of GJ organization and has implications for understanding how GJs form and how tissues regulate cell-cell interactions.

We never observed blurring of the bleach border produced within full-length Cx43 plaques, even when they were examined 1 h after the bleaching event. Since the average total time each Cx43 protein is part of the GJ plaque structure has been estimated to be less than 3 h (39-42), our results indicate that the arrangement of GJ channels within the plaque structure of Cx43 GJs does not change during the “in-plaque-lifetime” of the Cx43 channels. Thus, Cx43 “arrangement stability within the plaque” is not a relative term. We find that Cx43 forms statically arranged GJ plaques while Cx30 and Cx26 form highly fluidly arranged ones. Our findings that Cx43 is always statically arranged and Cx26 GJs are extremely fluid contrast with the observation of two distinct populations (static and dynamically arranged) for both Cx43 and Cx26 by Simek et al. (4). These differences may be due to experimental FRAP conditions or analysis methods, such as our 3D reconstructions of FRAP regions that corrects for whole-plaque movement that otherwise may be interpreted as dynamic plaque arrangement.

A curious aspect of statically arranged Cx43 GJ plaques is that substantial changes to overall shape of the plaque can occur while a sharp photobleach-generated border is maintained. This fits with the concept that Cx43 GJ-arrangement is maintained by abundant weak/transient interactions between neighboring channels while the overall plaque structure is malleable. Therefore, we hypothesize that this unusual plaque behavior is achieved through non-covalent lateral interactions between neighboring Cx43 channels or through binding to an extensive cytoplasmic scaffold. This idea is supported by our demonstration that plaque arrangement stability is completely eliminated when most of the cytoplasmic CT of Cx43 is removed. Simek et al. noted an increase in the percentage of mobile GJs for a different Cx43 truncation (Cx43 truncated at amino acid 244)(4). Using monomeric fluorescent proteins, we observed a qualitative switch from stable arrangement to fluid arrangement with truncation of Cx43 at either amino acid 258 or 245. This indicates that plaque stability is likely produced by a binding interaction of the Cx43 CT to COOH-termini of adjacent Cx43 channels or to a cytoplasmic scaffold. Truncation of the CT of Cx43 does reduce the molecular weight of the connexin from 43.03 kDa to 29.44 kDa. We show that the Cx43t258-msfGFP::Cx43-untagged plaques (estimated MW per channel= 602 kDa) are stably arranged while homotypic Cx43t258-msfGFP::Cx43t258-msfGFP (estimated MW per channel=687 kDa) form fluid plaques indicating that non-specific increased size is not the mechanism behind Cx43 stability (Figures 7,8) and as previously described for mobility characteristics of other membrane proteins(43,44).

We found that non-monomerized GFP can stabilize channel arrangement in normally fluidly arranged GJs. Taken together, the results of this experiments described here indicate that stability of Cx43 by the CT of the connexin is a separate endogenous stabilizing factor for GJ channel arrangement while, in contrast, stability imparted by CT-localized non-monomerized GFP is an experimental artifact. Fusion of the fluorescent fusion protein tag to the N-terminus of connexins prevents artificial stabilization. This likely occurs
due to the non-monomerized sfGFP no longer interacting with and weakly binding to sfGFP molecules attached to neighboring GJ channels. The NT of connexins are thought to be situated within the pore of GJs (35) while the CT are localized to the cytoplasm at a more medial position on the channel (illustrated in Fig. 5A,B drawings). Therefore, we propose that the sfGFP tagged onto the Cx26 NT (or Cx43K258stop NT) is repositioned away from sfGFP on neighboring GJ channels- thereby sterically blocked from binding- preventing GJ channel lateral clumping and plaque stabilization. The finding that placing the tag on the NT of connexins prevents stabilizing artifacts of GFP may be useful to rule out dimerization effects of other tags such as photoconvertible proteins that have been used in studies on GJ dynamics (7) and structure (8). It may also be important to test newly developed fluorescent proteins as tags on connexins by appending them to both the NT and separately to the CT of fluidly arranged connexins such as Cx26 and then comparing mobility with FRAP.

It should be noted that all normally-functional connexins so far tested with NT fluorescent protein tags form GJ plaque structures but do not allow dye or electrical coupling(45). This lack of channel function is thought to be due to the NT normally contributing to the GJ channel pore and the fluorescent protein tag at that location somehow disrupting functional channel formation (14,35,46). Therefore, NT tagged connexins cannot substitute for monomerized fluorescent protein CT connexin-tags when both “normal plaque structural dynamics” and some channel function are desired. Conversely, CT tags on connexins lead to altered gating, plaque size, and interactions with cytoplasmic binding partners such as ZO-1 (14,47). Loss of Cx43 interaction with ZO-1 has been shown to increase GJ plaque size. This appears to be a partial cause of the highly enlarged GJs seen in cultured cells over expressing connexins but is not likely the sole cause since we observed enlarged plaques in cells expressing NT-tagged and untagged Cx43. Cx43 tagged with EGFP on the NT has been shown to interact with ZO-1(48) and both full-length sfGFP-Cx43 and sfGFP-Cx43t258 over-expression led to abnormally large plaques in more than one cell type (Fig. 6, Table 4). In summary, we conclude that no fluorescent protein tagged connexin is optimal for every experimental design, that fluorescent protein tag type and location should be carefully considered for each particular experimental paradigm, and that the monomeric characteristics of a fluorescent protein can significantly impact the localization and dynamics of oligomeric integral membrane proteins.

Standard-resolution confocal light microscopy cannot resolve single GJ channels. Therefore, we cannot differentiate heteromorphic GJ channels within plaques from intermingled homotypic channels. Biochemical analysis techniques are better suited for these particular tests. Such work by others indicated that Cx43 and Cx30/Cx26 connexins do not form heteromeric channels (11,49) and consistent results were obtained for other similar connexin combinations (38). The high over-expression of connexins can saturate and overcome cellular control of connexin oligomerization(50). However, there was apparently enough control of Cx43 oligomerization in our experimental conditions to allow separate oligomerization when sfGFP dimerization produces segregated plaques. Additionally, saturation of oligomerization control does not account for differences produced by single amino acid changes to the fluorescent protein tag as shown in Fig. 9.

The finding that the position of the non-monomerized fluorescent protein tag affects mobility could indicate that mobility might be influenced by the length or flexibility of the linker. However, when monomeric fluorescent protein tags were used, similar mobility characteristics were observed for both stably arranged (Cx43) and fluid (Cx26) connexins regardless of the tag location- indicating that with improved fluorescent protein tags, tag location and consequently the size and sequence of the linker (within a reasonable range) do not substantially affect mobility.

A related caveat of the present study is that GFP and other fluorescent proteins are relatively large (5 nm) additions. The molecular weight of GFP is similar to Cx26 and Cx30 and nearly half the weight of Cx43. In the highly crowded environment of the GJ plaque, GFP might be expected to affect movement of GJ channels within the membrane. Indeed, the trafficking and assembly of connexins into GJs can be altered by the addition of fluorescent protein tags (5,47). We found that when tagged
and untagged connexins were mixed, the apparent dynamics of the resulting channel arrangement was not significantly affected. Heteromeric tagged:untagged Cx26 and Cx43-truncation constructs exhibited significantly increased mobility compared to full length Cx43. These results also established that fluidity observed for Cx26, Cx30, and Cx43K258stop is unrelated to the fluorescent protein tag. The experiments with tagged:untagged connexins indicate that FRAP with msfGFP tagged connexins can reveal the characteristics of untagged connexins (at least in the case of transgenic overexpression, see next section).

The abnormally large plaques formed by over-expression of connexins in cultured cells contrasts with the smaller plaques that form in vivo [~0.01-2.0 µm (51)]. However, the dynamic characteristics we uncovered here are expected to be relevant for in vivo GJ structure since the arrangement stability of Cx43 channels was dependent on amino acids within the CT of Cx43 and was maintained by Cx43 without a fluorescent protein tag (Figures 7,8). Stability is an intrinsic property of Cx43 channels and not present in GJs composed of Cx26 and Cx30. In addition, the mobility characteristics we report were features of GJs ranging from 4 µm to more than 20 µm in diameter and were maintained at both the center and perimeter of the plaques (Fig. 3D, and 3D reconstructions shown in Supplemental Movies 2-6). In spite of these caveats, our results provide new insights into GJ arrangement-stability and plaque structure and how these properties are modified by connexin type, fluorescent-protein tag type, and tag location. Our findings also indicate that the CT is required for formation of stable Cx43 GJ plaques.

We also conclude that the sharp segregation of Cx43 and Cx26 within the same plaque reported previously was a result of aggregation of CT, non-monomerized tags. Non-mixed GJ plaques readily form when non-monomerized tags are used (Fig. 9A,B, and (6)). To potentially explain the underlying mechanism, we considered all of the different combinations of tag location and tag type examined in this study (Table 4.), and we formulated the following hypothesis. We propose that strong lateral interactions are needed to form non-intermingled GJs as with sfGFP dimerization in EBFP2-Cx43 + Cx26-sfGFP GJs (Fig. 9A,B) and an imbalance is required between the strength of lateral interactions between the two Cx-tag types that are present in the GJ. We hypothesize that clustering according to Cx-tag type occurs as new GJ channels form at the edge of the plaque. If the drive to cluster is strong (as with Cx26-sfGFP) this strong clustering “squeezes out” the weaker lateral interactions of the Cx43 channels- at the very local level of the plaque periphery. Once the channels are fully clustered into the plaque structure they would be locked into their arrangement by the stably arranged Cx43, according to this scenario. If this hypothesized mechanism behind intermingled and non-intermingled plaques is correct then segregated plaques could form in vivo if Cx43 expression preceded that of Cx26 or Cx30 expression. Therefore, the present study does not rule out formation of unmixed GJ plaques in vivo, but we do show that non-monomerized fluorescent proteins strongly interfere with tests aimed at investigating this intriguing model.

NT-tagged connexins might be most appropriate for future studies aimed at examination of connexin plaque arrangement stability and/or arrangement of binding partners at the GJ plaque. NT-tagged connexins have proven useful in this regard in previous studies (48,52-55). If channel activity is required, the msfGFP CT tags are likely the current best choice.

The connexins examined here (Cx43, Cx30 and Cx26) are co-expressed in astrocytes in the mammalian brain. Each connexin type has different channel properties, binds unique sets of cellular proteins, and has different regulation of expression, trafficking, and activity. Therefore, our results will be important as we achieve finer resolution mapping of astrocyte connectivity within the brain. This study demonstrates that mobility and arrangement of Cx26 and Cx30 are affected by fluorescent protein tag type and this finding should be taken into account for studies using fluorescent protein fusion constructs as localization markers in highly crowded and ordered cellular compartments. Several recent reports have indicated that the arrangement of certain phosphorylated-Cx43 GJs is regulated and spatially segregated within the plaque (18,19). We hypothesize that the stability that we observed for Cx43 plaques is important for localization of specific posttranslational modification of GJs to
appropriate sub-regions of Cx43 GJ plaques. This raises the exciting possibility that cells express multiple connexin types to allow differential GJ arrangement stability. Cx43 and Cx30 were recently shown to have differential tissue distribution in the mouse visual cortex and that this distribution is correlated with functional organization of cortical brain tissue, at least in layer IV of this region of the cerebral cortex (56). Deletion of astrocyte Cx30 alone or both Cx43 and Cx30 were shown to have opposite effects on synaptic transmission and plasticity (57,58). These differences in neuronal activity were ascribed to channel independent effects of the Cx30 CT. Additionally, Cx43 and Cx30 are highly localized to the astrocyte process that wraps specifically around brain vasculature (known as the astrocyte endfoot domain). Other integral membrane proteins such as AQP4 and Kir4.1 are highly localized to the same area where Cx43 and Cx30 plaques cluster in the endfoot. Localization of AQP4 and Kir4.1 to the astrocyte endfoot has been shown to be important in the pathological manifestation of mouse models of neural disease and human brain samples (59-65). We speculate that different properties imparted to the endfoot and peri-synaptic domains of astrocytes by stable or fluid GJ plaques affect astrocyte morphology and localization of other proteins that are required for brain homeostasis including AQP4 and Kir4.1. Alterations to astrocyte connexin expression and localization have been reported for most neurological diseases (60,64,66-70). Results we present in this study may be important for improving our understanding of how alteration to astrocyte connexin expression, localization, and function fit into the etiology of complex neurological disorders.

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CONFLICTS OF INTEREST
A provisional patent application has been filed covering part of the results described in this manuscript (E.L.S). Albert Einstein College Medicine and E.L.S. have licensed technology described in this manuscript to Lucigen Corp.

AUTHOR CONTRIBUTIONS
RFS performed the experiments, analyzed the experiments, and prepared the figures. ELS and DCS provided technical assistance and contributed to experimental design and analysis. All authors helped conceive the study and design the experiments. All authors wrote the manuscript and contributed to preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.
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Gap junction plaque organization

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FOOTNOTES

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2 Department of Anatomy & Structural Biology, Albert Einstein College of Medicine, 1410 Pelham Parkway South, Bronx, NY 10461, USA.
3 The abbreviations used are: CT, COOH-terminus; Cx, connexin; Cx26, connexin 26; Cx30, connexin 30; Cx43, connexin 43; Cx43t258, Cx43 truncated at amino acid 258, DMEM, Dulbecco’s Modified Eagle Medium; EBFP2, enhanced blue fluorescent protein 2, ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; GJ, gap junction; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mVenus, monomeric venus fluorescent protein; msfGFP, monomerized superfolder green fluorescent protein; NA, numerical aperture; N2A, Neuro2a; NT, NH2-terminus; PBS, phosphate buffered saline; RINm, Rat Insulinoma; ROI, region of interest; RT, room temperature; sfGFP, non-monomerized superfolder green fluorescent protein.

Recovery was never observed in FRAP experiments with RINm cells expressing sfGFP-Cx43 with average percent normalized recovery of 9.3±4.7% at 30 s post bleach in 7 experiments, this small apparent recovery in single plane FRAP experiments was an artifact of whole plaque movement since no recovery was ever observed when 3D reconstructions acquired 2 min post-bleach were examined.

Results for these heterotypic FRAP experiments were validated in 1-2 trials each, in N2A cells using CellTracker Orange CMTMR loaded into the cells that received transfection of untagged connexin expression plasmids. The cells labeled with CellTracker dye were then washed and the cells were replated with cells transfected with msfGFP tagged connexin. This avoids any possible interaction of the untagged connexin with the fluorescent protein as there was none in the N2a cells expressing untagged connexin.

Cx43-sfGFP co-expressed with EBFP-Cx30 led to separated clusters of Cx43-sfGFP that were smaller and more numerous than those observed when Cx30-sfGFP was co-expressed with EBFP2-Cx43. In the four GJs where Cx43-sfGFP appeared to intermingle with EBFP2-Cx30 there appeared to be very small clusters of Cx43-sfGFP below the resolution of the microscope (~250 nm).

Some GJ plaques (4/15) made up of untagged Cx43 and Cx26-msfGFP had small areas at the edge of the plaque with only green (Cx26-msfGFP) signal, but in all of these four cases, the rest of the adjacent plaque area next to these small areas were always well mixed for the two colors.
FIGURE LEGENDS

FIGURE 1. Characterization of FRAP experiments on GJ plaques. A, Percent of the fluorescence pool that is bleached (fraction of the plaque within the 2D confocal plane bleached) is larger than is standard for FRAP experiments on membrane proteins. This is due to several factors: i) a plaque is effectively a one dimensional structure although the majority of the fluorescence pool is out of the focal plane, ii) The plaque size (true fluorescence pool available for recovery on the time scale of <2 min) is variable. iii) Any tilt in the plaque orientation away from parallel to the light path will alter the shape of the intersection of the bleaching laser with the plaque in out of focus regions of the plaque. iv) Reduction of the size of the bleach region below 2 µm would lead to drastically increased error due to cell shape changes (whole plaque movement), this form of error has an intrinsic bias towards recovery since any movement of the plaque will lead to less thoroughly bleached areas of the plaque moving into the focal plane. B, Maximum percent change in fluorescence pool average intensity (normalized to post-bleach fluorescent pool intensity) over 60 s was not significantly different for the Cx-msfGFP groups. This indicates that whole plaque movement and the combined effect of out-of-focus bleach and arrangement fluidity were, altogether, not significantly different. No significant difference was seen in any groups in these figures as analyzed by ANOVA followed by Tukey’s multiple comparison. C, Time-lapse images of a Cx30-msfGFP expressing cell-pair in which two separate plaques were formed side-by-side. ROIs were created around the region of the plaque to be bleached (ROI 1), around the plaque to be bleached (ROI 2, fluorescence pool), around the plaque that did not have any part bleached (ROI 3), and a part of the cell without GJ plaque (ROI 4, background). White scale bar=5 µm. D, 3D reconstructions of the plaques in “C”. Pre-bleach (left), and 2 min post-bleach (right) the plaque that was bleached is less bright all over because bleached Cx43-msfGFP has redistributed throughout the plaque area. The plaque on the left changes shape but overall brightness changes very little during the FRAP experiment in “C”. White scale bar=2 µm. E) Non-normalized traces showing the average fluorescence intensities for each of the ROIs in “C”. Green= ROI 1=Bleach region, the bleach event creates a step down in intensity at 10 s, relatively minor apparent recovery is due to large percent of fluorescence pool being bleached. Normalization corrects this error in FRAP curves. Blue= ROI 2=Fluorescence pool, the step-down at Gray=ROI 3=Un-bleached plaque, little change over 1 min indicates minor acquisition bleach. Red=ROI 4= Background, Minimal change in background is observed.

FIGURE 2. Examples of border analysis for Cx43-msfGFP (A-C) and Cx30-msfGFP (D-F). A,D, Time-lapse image of a Cx-msfGFP plaque before, 0.6 s post-bleach, and 30 s post-bleach. Yellow box indicates region used for a 5 pixel high (5 X averaged line scan) and 20 pixels wide analysis. Scale bar = 5 µm. Pixel size 0.2 µm. B,E, 5X averaged line scan across the bleach border smoothed 3X. C,F. Absolute value of the differential at each point along the curves from “B and E”. The maximum value for 30 s post-bleach was subtracted from the maximum value from 0.6 s post-bleach and this value was normalized by dividing it by the 0.6 s post-bleach maximum to get change (dF) of initial value (Fo) and this fraction was expressed as a percent. This operation was performed on the image data from each FRAP experiment used in bleach border analysis and the dF/F0% (percent border blur at 30 s) was expressed as mean ±SEM in Figures 3-8.

FIGURE 3. GJ plaque arrangement dynamics depend on connexin type. A-D. Single plane confocal time-lapse images showing, from left to right: pre-bleach, 0.6 s after photobleach, and 2 min after photobleach. A line scan (from the area of the GJ plaque indicated by the yellow bracket) shows the intensity profile of the bleach region to illustrate recovery or lack thereof. A, Cx43-msfGFP GJs do not rearrange their respective positions within the plaque as indicated by the maintenance of a sharp bleach border (steep transition in intensity at edge of bleach) at 2 min. B, Cx26-msfGFP GJ plaques are very dynamically arranged as indicated by significant movement of unbleached Cx26-msfGFP into the bleach region and blurring of the bleach border. C, Cx30-msfGFP plaques are also dynamically arranged. D,
Cx43-msfGFP GJ arrangement is static for > 1 h. Left panel: single-plane confocal image of a pair of HeLa cells joined by a plaque aligned with the axis of the light path. Right panels: top row shows a maximum-projection reconstruction of the plaque at each time point with a single plane image shown below. E, Normalized percent recovery (%FRAP) over time for each connexin shown in A-C. n=11,10, 12 for Cx43, Cx26 and Cx30, respectively. F, Recovery (% normalized recovery of fluorescence into the bleach region) of Cx43 is significantly less than both Cx26 and Cx30 (in both HeLa cells and N2A cells) as analyzed by ANOVA followed by Tukey’s multiple comparisons. G, Bleach border blurring for Cx43 is significantly less than both Cx26 and Cx30 as analyzed by ANOVA followed by Tukey’s multiple comparisons. ** indicates p<0.01, *** indicates p<0.001. Number of GJs analyzed is written next the bar in bar graphs, all error bars SEM, scale bars in A-D= 5 µm.

**FIGURE 4.** Fluorescent protein tag dimerization blocks mobility of otherwise dynamically arranged Cx26. A, Cx26-sfGFP plaque arrangement is static. Single plane confocal images before, 0.6 s after, and 2 min after photobleach. Scale bar=5µm. B, Averaged normalized recovery for Cx26-msfGFP (top trace, sourced from Fig.3), and Cx26-sfGFP. Cx26-sfGFP and Cx26-msfGFP n values = 7 and 10, respectively. C, Cx26 tagged with non-monomerized sfGFP (sfGFP) has significantly less recovery at 30 s (average percent recovery) compared to Cx26-msfGFP and Cx26-mVenus (mVenus has a monomerizing lysine at position 206). D, Border analysis also indicates a significant reduction in plaque arrangement fluidity at 30 s post bleach for Cx26 tagged with original sfGFP compared to Cx26-msfGFP and Cx26-mVenus. All error bars are SEM. Data for Cx26-msfGFP sourced from Fig. 3. Groups compared by ANOVA followed by Tukey’s Multiple Comparisons Test. Number of experiments is listed on each bar of the graphs. ** indicates p<0.01, *** indicates p<0.001. Non-monomerized fluorescent protein tag has a similar effect on Cx30 but does not affect Cx43.

**FIGURE 5.** Translocation of original sfGFP from the CT of Cx26 to the NT occludes the stabilizing effect of tag dimerization. A, Illustration of a single Cx26 protein (brown) tagged with sfGFP on the COOH-terminus (CT) on the left, and a side-view illustration of a small plaque with GJs spanning the membranes (gray lines) of adjacent cells. Note the fusion of GFP to the CT of the connexin positions it near the outside of the channel (within the cytoplasm of the cells), which may allow it to dimerize with GFP attached to a neighboring different channel. B, Illustration showing that when the GFP is attached to NH2-terminus (NT) of the Cx26 it is likely positioned more closely to the channel pore and away from neighboring channels. C, Plaques made up of sfGFP-Cx26 are not stably arranged. Single plane confocal images before, 0.6 s post-bleach, and 2 min after photobleach. D, Averaged normalized recovery for Cx26-msfGFP (green top trace, sourced from Fig.3), and Cx26-sfGFP (black/green bottom trace sourced from Fig. 4) and sfGFP-Cx26 (blue, n=13). E, Cx26 tagged with non-monomerized sfGFP (sfGFP) on the NT has significantly more recovery at 30s (Average percent recovery) compared to Cx26-msfGFP, there was no significant difference between sfGFP-Cx26 and Cx26-sfGFP. F, Border analysis also indicates that the bleach-induced border blurs significantly more when the sfGFP is attached to the NT than when it is on the CT, at 30 s post bleach. G, Cx43 forms stably arranged GJ plaques regardless of tag type and tag location. All error bars are SEM. Groups compared by ANOVA followed by Tukey’s Multiple Comparisons Test. Number of experiments per group indicated above the bar graph for each group. * indicates p<0.05, ** indicates p<0.001. Cx43 plaque arrangement stability is not affected by non-monomerized fluorescent protein tag. Some data for G is sourced from Figure 3.

**FIGURE 6.** Cx43 plaque arrangement stability requires the cytoplasmic CT. A, Cx43 truncated at amino acid 258 forms GJ plaques (left panel). GJ plaques made up of Cx43K258stop-msfGFP are fluidly arranged as indicated by movement of unbleached fluorescent protein tagged Cx43t258 into the bleached region (Cx43K258stop-msfGFP in HeLa cells shown in this example, scale bar = 5 µm). A line scan (from the area of the GJ plaque indicated by the yellow brackets) shows the intensity profile of the bleached region to illustrate recovery. B, Normalized FRAP curves indicate a difference between full-length Cx43 and Cx43 truncated at amino acid 258, Orange trace = Cx43t258-msfGFP in HeLa cells n=3,Upper gray
FIGURE 7. Presence of a monomerized fluorescent protein tag does not qualitatively alter GJ plaque arrangement stability. A, Two separate HeLa cell cultures were transfected then replated together. The image shows an example of pairs of cells in which Cell 1 and Cell 2 expressed Cx43t258-msfGFP. Cell 3 expressed mCherry marking it as a cell from the culture that was transfected with full-length rat Cx43 without a fluorescent protein tag. This panel shows a single plane of a 3D Z-stack performed 2 min after 2 μm of both the top and bottom plaques were bleached. The top plaque made up of homotypic Cx43t258-msfGFP has recovered fluorescence signal in the bleached region but the un-tagged full-length Cx43 expressed by Cell 3 has stabilized the heterotypic, lower plaque - thereby preventing recovery into the bleached region. Scale bar= 5 μm. B, Cartoon to clarify the position of cells and forms of Cx43 making up the plaque (not to scale). C, Heterotypic tagged::untagged plaques are significantly dimmer than tagged::tagged plaques. D, Heterotypic tagged::untagged (Cx26-msfGFP::Cx26) plaques are significantly dimmer than homotypic Cx26-msfGFP::Cx26-msfGFP plaques. E, FRAP recovery curves for heterotypic Cx43t258-msfGFP::untagged-Cx43 (red circle-markers) and heterotypic Cx26-msfGFP::Cx26 (green circle-markers). n=6 and 3 for Cx43 and Cx26, respectively. F and G, Untagged Cx43 stabilizes the arrangement of channels within the GJ and untagged Cx26 is fluidly arranged within heterotypically-tagged GJs. * indicates p<0.05, ** indicates p<0.01. All groups in C-D were compared by Student’s unpaired T-test. Groups in E-G were compared by one-way ANOVA followed by Tukey’s multiple comparisons. Data for homomeric-tagged channels in F and G is sourced from Figure 3.

FIGURE 8. Mobility of untagged connexins in heteromeric complexes is similar to that of tagged connexins. A, Percent FRAP at 30s for heteromeric tagged/untagged GJs is similar to GJs formed in cells only transfected with tagged connexins as in Figs. 3 and 6. Data for homomeric msfGFP tagged connexins is sourced from Figure 3. B, Bleach border analysis indicates similar amount of GJ channel rearrangement for GJ plaques only partially tagged (a.k.a. heteromeric-tagged) is similar to fully-tagged (a.k.a. homomeric-tagged, as in Figs. 1-5). C, Percent recovery at 30 s is not correlated to plaque intensity for heteromeric Cx43t258-msfGFP/ Cx43 plaques (even when potential outlier is removed) indicating that only a small percentage of channels required an intact CT in heteromeric (truncated/full-length) plaques to form a stable channel arrangement. Note data points shown in C are from a different experimental group than represented in A and B.

FIGURE 9. Fluorescent protein tag dimerization is the cause of large-scale segregation of GJs within mixed-type plaques. A, When Cx26-sfGFP (non-monomerized FP tag) is co-expressed with EBFP2-Cx43 the two types of GJs are segregated to nearly mutually exclusive domains. B, When Cx30-sfGFP is expressed with EBFP2-Cx43 it also forms Cx type-specific, large clusters. C, When Cx26 tagged with monomerized sfGFP is co-expressed with EBFP2-Cx43 the two types of GJ intermingle within the plaque (but not in the Golgi/ER complex, shown in Supplemental Movies. D, Replacing sfGFP with msfGFP as the tag on Cx30 has a similar intermingling effect on the arrangement of plaques made up of Cx30 and EBFP2-Cx43. E-F, Re-locating the original sfGFP from the CT to the NT of Cx30 and Cx26 has a similar effect on plaque intermingling with EBFP2-Cx43 as it does on mobility likely due to relocation towards the pore of the GJ channel and prevention of the sfGFP to dimerize with a sfGFP tag on a neighboring channel. G, Non-monomerized Cx26-sfGFP forms separate regions within Cx43/Cx26 mixed plaques in
N2A cells. H, When Cx26-msfGFP and EBFP2-Cx43 are co-transfected into N2A cells the resulting plaques are well mixed with respect to connexin type. Images in A-F are of HeLa cells, images G and H are of N2A cells. Scale bars in A,B,G,H =2 µm, scale bars in C-F =5 µm.

**FIGURE 10.** Fluorescent protein clustering and intermingling without fluorescent protein tags on one or both connexins. A, When immunostained-Cx43 and Cx26-sfGFP are localized to the same plaque they are segregated to mostly non-overlapping domains. B, When untagged Cx43 is co-expressed with Cx26-msfGFP, however, more intermixing takes place although some regions with more or less Cx26 can be observed (yellow arrows). C, Intermixing of untagged Cx26 and Cx43. D, Intermixing of untagged Cx26 and Cx43 is also observed in N2A cells over-expressing untagged connexins. Red=Cx43 (Alexa594-labeled secondary). Green = Cx26-GFP in A and B, and Alexa488-labeling of Cx26 in C and D of this figure. E, When NT tagged Cx30 (EBFP2-Cx30) is co-expressed with Cx43-sfGFP the two GJ types are localized to separate regions within the plaque. Interestingly, in this case the Cx43-sfGFP is localized to the center of the plaque whereas Cx43 localizes throughout the plaque when EBFP2-Cx43 is co-expressed with Cx26-sfGFP or Cx30-sfGFP. F, When EBFP2-Cx30 is co-expressed with Cx43-msfGFP the channels intermingle, but there may be some Cx43-msfGFP grouping that occurs leading to small clustered structures near the resolution limit of light microscopy. Images in A-C,E-F are of HeLa cells, Images in D are of N2A cells. Scale bar=2 µm.
### Table 1. Summary of main FRAP results

| Connexin and Tag   | Expected topology at GJ plaques | Stable or Fluid channel arrangement | Figure  | Cell types tested    |
|--------------------|--------------------------------|------------------------------------|---------|----------------------|
| Cx26-msfGFP        | ![Fluid](image)                | Fluid                              | Fig. 3  | HeLa, N2A, RINm      |
| Cx30-msfGFP        | ![Fluid](image)                | Fluid                              | Fig. 3  | HeLa, N2A            |
| Cx43-msfGFP        | ![Stable](image)               | Stable                             | Fig. 3  | HeLa, N2A            |
| Cx43K258stop-msfGFP| ![Fluid](image)                | Fluid                              | Fig. 6  | HeLa, N2A            |
### Table 2. Additional FRAP results- effects of protein tag and tag location

| Connexin and Tag                  | Expected topology at GJ plaques | Stable or Fluid channel arrangement | Figure  | Cell types tested |
|----------------------------------|---------------------------------|-------------------------------------|---------|-------------------|
| Cx26-sfGFP                       | ![Diagram](http://www.jbc.org/) | Stable                              | Fig. 4  | HeLa              |
| sfGFP-Cx26                       | ![Diagram](http://www.jbc.org/) | Fluid                               | Fig. 5  | HeLa              |
| Cx30-sfGFP                       | ![Diagram](http://www.jbc.org/) | Stable                              | Fig. 4  | HeLa              |
| sfGFP-Cx30                       | ![Diagram](http://www.jbc.org/) | Fluid                               | Fig. 5  | HeLa              |
| sfGFP-Cx43                       | ![Diagram](http://www.jbc.org/) | Stable                              | Fig. 5  | HeLa, N2A, RINm   |
| msfGFP-Cx43                      | ![Diagram](http://www.jbc.org/) | Stable                              | Fig. 5  | HeLa              |
| Cx43K258stop-sfGFP               | ![Diagram](http://www.jbc.org/) | Stable                              | Fig. 6E | HeLa, N2A         |
| sfGFP-Cx43K258stop               | ![Diagram](http://www.jbc.org/) | Fluid                               | Fig. 6E | HeLa, N2A         |
Table 3. Additional FRAP results - un-tagged connexins to test for more endogenous-like behavior.

| Connexin and Tag                        | Expected topology at GJ plaques | Stable or Fluid channel arrangement | Figure | Cell types tested |
|----------------------------------------|---------------------------------|-------------------------------------|--------|-------------------|
| Heterotypic Cx43 + Cx43K258stop-msfGFP | Stable                          | Fig.7                               | HeLa, N2A                      |
| Heterotypic Cx26 + Cx26-msfGFP         | Fluid                           | Fig.7                               | HeLa, N2A                      |
| Heteromeric Cx43 + Cx43-msfGFP         | Stable                          | Fig.8A, B                           | HeLa                                  |
| Heteromeric Cx26 + Cx26-msfGFP         | Fluid                           | Fig.8A, B                           | HeLa                                  |
| Heteromeric Cx43S257stop + Cx43K258stop-msfGFP | Fluid | Fig.8A, B                           | HeLa                                  |
| Heteromeric Cx43S257stop + Cx43-msfGFP | Stable                          | Fig.8C                              | HeLa                                  |
Table 4. Summary of observations on Heterotypic intermingling of co-expressed connexins.

| Connexins co-expressed | Expected channel topology drawing | Non-mixed plaques / plaques examined | Avg. diameter of GJs examined (µm) |
|------------------------|-----------------------------------|-------------------------------------|----------------------------------|
| EBFP2-Cx43 + Cx26-sfGFP | ![topology drawing](image1)         | 9/9 HeLa                           | 5                                |
|                        |                                   | 6/6 N2A                             | 9                                |
| EBFP2-Cx43 + Cx26-msfGFP| ![topology drawing](image2)       | 0/3 HeLa                           | 7                                |
|                        |                                   | 0/6 N2A                             | 8                                |
| EBFP2-Cx43 + Cx30-sfGFP | ![topology drawing](image3)       | 5/5                                 | 6                                |
| EBFP2-Cx43 + Cx30-msfGFP| ![topology drawing](image4)       | 0/7                                 | 5                                |
| EBFP2-Cx43 + sfGFP-Cx26 | ![topology drawing](image5)       | 0/8                                 | 6                                |
| EBFP2-Cx43 + sfGFP-Cx30 | ![topology drawing](image6)       | 0/4                                 | 10                               |
| EBFP2-Cx26 + Cx43-sfGFP | ![topology drawing](image7)       | 6/7                                 | 9                                |
| EBFP2-Cx30 + Cx43-sfGFP | ![topology drawing](image8)       | 13/17 c)                           | 7                                |
| EBFP2-Cx30 + Cx43-msfGFP| ![topology drawing](image9)       | 0/9                                 | 8                                |
| untaggedCx43 + Cx26-sfGFP | ![topology drawing](image10)     | 15/18                               | 8                                |
| untaggedCx43 + Cx26-msfGFP | ![topology drawing](image11)     | 0/15 d)                             | 10                               |
| untaggedCx43 + untaggedCx26 | ![topology drawing](image12)     | 0/5 HeLa                           | 4                                |
|                        |                                   | 1/8 N2A                             | 6                                |
A. Fluorescent Pool Bleached

B. Max Fluorescent Pool Change After First Bleach Time-point

C. Before Post-Bleach 30s 60s

D. Pre-Bleach 2 min Post-Bleach

E. Average Intensity From Expt. Above

- Unbleached plaque (ROI 3 in C)
- Bleach ROI (ROI 1 in C)
- Fluorescence Pool (Fp, ROI 2 in C)
- Background (ROI 4 in C)
Stout et. al. Figure 2.

A. Cx43-msfGFP
   Pre-bleach
   Post-bleach
   30s post-bleach

B. Line-scan on Border
   Differential (Abs. Val.)

C. Differential of Line-scan
   dF/Fo=3.1%

D. Cx30-msfGFP
   Pre-bleach
   Post-bleach
   30s post-bleach

E. Line-scan on Border
   Differential (Abs. Val.)

F. Differential of Line-scan
   dF/Fo=65.1%
Stout et al. Figure 4.

A. Pre-bleach  Post-bleach  120s Post-bleach

B. Intra-plaque FRAP

C. FRAP at 30s

D. Border Blur at 30s
Stout et. al. Figure 5.

A. Cx26-sfGFP

inter-gap junction dimers stabilize

B. sfGFP-Cx26

intra-gap junction dimers allow fluidity

C. Pre-Bleach Post-bleach 120s Post-bleach

sfGFP-Cx26

D. Intra-plaque FRAP

Normalized recovery (%)

E. FRAP at 30s

Normalized recovery (%)

F. Border Blur at 30s

Normalized recovery (%)

G. FRAP at 30s

Normalized recovery (%)
Stout et. al. Figure 6.

A. Before 0.6s Post-bleach 120s Post-bleach

B. Normalized recovery (%)

C. FRAP at 30s

D. Border Blur at 30s

E. FRAP at 30s HeLa and N2A Combined

Cx43-msfGFP

Cx43t258-msfGFP

N2A cells

N2A cells truncated

line-scan A.U.

HeLa and N2A Combined

Normalized % FRAP at 30s

Normalized % FRAP at 30s

Normalized % FRAP at 30s

Normalized % FRAP at 30s

Normalized % FRAP at 30s

A.U.

A.U.

A.U.

A.U.
Stout et. al. Figure 7.
Stout et al. Figure 8.

A. FRAP at 30s

B. Border Blur at 30s

C. Heteromeric Cx43t258-msfGFP + Cx43-NoTag

Plaque Intensity (A. U.)

Normalized FRAP at 30s

Border Blur at 30s

Recovery at 30s (%)

FRAP at 30s

Border Blur at 30s

Recovery at 30s (%)
Stout et al. Figure 10.

A. Cx26  Cx43  Overlay
   Cx26-sfGFP  Cx43  Cx43 (no tag)  Cx43
   Cx26 msfGFP

B. Cx26-sfGFP
   Cx43 (no tag)
   Cx26 Cx43
   Cx26 (no tag)

C. Cx26 (no tag)
   Cx43 (no tag)
   Cx26  Cx43

D. Cx26 (no tag)
   Cx43 (no tag)
   Cx26  Cx43
   Cx26 Cx43 Overlay

E. EBFP2-Cx30  Cx43-sfGFP  Overlay

F. EBFP2-Cx30  Cx43-msfGFP  Overlay

Stout et al. Figure 10.
Connexin Type and Fluorescent Protein-fusion Tag Determine Structural Stability of Gap Junction Plaques
Randy F. Stout, Jr., Erik Lee Snapp and David C. Spray

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