SI Appendix

Cell Lines and culture conditions. Experiments were performed in HeLa cells transfected with wild type Cx43 and Cx45 and their fusion forms with color variants of green fluorescent proteins (EGFP or CFP) tagged to the C-terminus of Cxs. Vectors used for transfection and cell lines stably expressing examined Cxs were developed in collaboration with the laboratories of Dr. D.W. Laird. (Cx43), Dr. K. Willecke (Cx45-CFP), and Dr. T.A. Bargiello (Cx45 and Cx45-EGFP). More details on these issues are reported earlier (1, 2, 3). To study heterotypic junctions, cells expressing different connexins were seeded on coverslips. Isolated heterotypic cell pairs were selected by identifying those in which the two cells were expressing GFPs differing in color or in which one cell expressed GFP and other was labelled with DAPI or DiI.

Electrophysiological Measurements. Junctional conductance (g_j) was measured using a dual whole-cell voltage clamp system. Briefly, each cell of a pair was voltage clamped independently with a separate patch clamp amplifier (EPC-7plus; HEKA). By stepping the voltage in one cell (ΔV_1) and keeping the other constant, junctional current (I_j) was measured as the change in current in the unstepped cell, $I_j=-\Delta I_2$; I_j has always the same polarity as the voltage step in cell 1. Thus, g_j was obtained from the equation $g_j= I_j/(V_1-V_2)$.

Fluorescence imaging and dye transfer studies. Fluorescence signals were acquired using an ORCA digital camera (Hamamatsu Corp., Bridgewater, NJ) with UltraVIEW software for image acquisition and analysis (Perkin Elmer Life Sciences, Boston, MA). For dye transfer studies, a given dye was introduced into cell-1 of a pair through a patch pipette in whole-cell voltage clamp mode. Dyes used include (molecular mass of the fluorescent ion, valence): Alexa Fluor-350 (AF^{350}) (326, -1) and Lucifer yellow (LY) (443, -2) (Invitrogen, Eugene, OR). Typically, breaking into cell-1 resulted in a rapid loading of cell-1 followed by dye transfer to cell-2. In our earlier studies of single channel permeability of GJ channels, a whole cell recording in the dye recipient cell (cell-2) was established ~6-10 min after opening the patch in cell-2. This allowed us to measure g_j and avoid dye loss due to its diffusion from cell-2 to the patch pipette. The nature of the present studies required us to examine dye transfer by using combined fluorescence imaging and g_j measurements; therefore, we needed to account for a dye loss from pipette-2.

For estimation of junctional permeability (P_j) we used a modified Goldman-Hodgkin-Katz (GHK) equation (4) adapted for P_j studies (5). The transjunctional flux (J_j) of dye through gap junction channels, when both patch pipettes are in the whole-cell recording mode, can be determined by the change in dye concentration in cell-2 (ΔC_2) over the time interval (Δt) and the leakage of dye to patch pipette-2 as follows,

$$J_j = \left(vol_2\, \Delta C_2 / \Delta t\right) + P_p C_2 \qquad \#1$$

where vol_2 is the volume of cell-2 and P_p is the permeability characterising dye leakage from cell-2 to pipette-2 (see equation #11). This definition of J_j is different from the classical one, which defines the flux per unit area. In our studies and those of Verselis et al. (5), the cross-sectional area of the gap junction is unknown and difficult to estimate. Consequently, we expressed total fluxes in mol/s and total permeabilities in cm^3/s, and normalized J_j and P_j to g_j or number of open channels computed as g_j divided by single channel conductance, γ.

Assuming that the Goldman-Hodgkin-Katz (GHK) current equation (4) applies to J_j, then J_j can be expressed through the total junctional permeability (P_j) multiplied by the driving force, which for a charged molecule involves both the concentration and voltage gradients (V_j):

$$J_j = \frac{P_j \left(zFV_j/RT\right) \left[C_1 - C_2 \exp\left(-zFV_j/RT\right)\right]}{\left[1 - \exp\left(-zFV_j/RT\right)\right]} \quad \#2$$

where z is the net charge of the dye molecule, F is Faraday's constant, V_j is the transjunctional voltage, R is the gas constant, T is the absolute temperature, and C_1 and C_2 are dye concentrations in cell-1 (dye-donor) and cell-2 (dye-recipient), respectively. In the absence of a voltage difference equation #2 reduces to:

$$J_{j,0} = P_{j,0} \left[C_1 - C_2\right] \quad \#3$$

where $J_{j,0}$ and $P_{j,0}$ are J_j and P_j at V_j=0 mV, respectively. Based on our studies as well as others (7,8), we assumed that when the concentration of dye is below 1 mM, dye concentration (C) is directly proportional to fluorescence intensity (FI) measured in arbitrary units (a.u.), C=k(FI), where k is a constant. Then, equation #1 can be express as follows:

$$J_j = k\left[\left(vol_2 \, \Delta FI_2/\Delta t\right) + P_p \, FI_2\right] \quad \#4$$

where $\Delta FI_2 = FI_{2(n+1)} - FI_{2(n)}$ is the change in FI in cell-2 over the time, $\Delta t=(t_{n+1}-t_n)$; n is n^{th} time point in the recording. Consequently equation #2 and #3 will be as follows:

$$J_j = \frac{P_j \, k \left(zFV_j/RT\right) \left[FI_1 - FI_2 \exp\left(-zFV_j/RT\right)\right]}{\left[1 - \exp\left(-zFV_j/RT\right)\right]} \quad \#5$$

$$J_{j,0} = P_{j,0} \, k \left[FI_1 - FI_2\right] \quad \#6$$

Expressions for P_j and $P_{j,0}$ can be found from equations #5 and #6, respectively, as previously shown in Verselis et al. (5), and substituting J_j and $J_{j,0}$ by their expressions:

$$P_j = \frac{\left[\left(vol_2 \, \Delta FI_2/\Delta t\right) + P_p \, FI_2\right] \left[1 - \exp\left(-zFV_j/RT\right)\right]}{\left(zFV_j/RT\right)\left[FI_1 - FI_2 \exp\left(-zFV_j/RT\right)\right]} \quad \#7$$

$$P_{j,0} = \frac{\left[\left(vol_2 \, \Delta FI_2/\Delta t\right) + P_p \, FI_2\right]}{\left[FI_1 - FI_2\right]} \quad \#8$$

To measure how V_j affects J_j, we can determine the ratio of J_j to $J_{j,0}$, so that:

$$\frac{J_j}{J_{j,0}} = \frac{P_j}{P_{j,0}} \frac{\left(zFV_j/RT\right)\left[FI_1 - FI_2 \exp\left(-zFV_j/RT\right)\right]}{\left[1 - \exp\left(-zFV_j/RT\right)\right]\left[FI_1 - FI_2\right]} \quad \#9$$

If we assume that $P_j/P_{j,0} = g_j/g_{j,0}$ for neutral molecules, when $g_{j,0}$ is the junctional conductance measured at $V_j \approx 0$ mV, we can use g_j-V_j measurements to predict how $J_j/J_{j,0}$ should change for neutral molecules in response to V_j and compare that with the experimentally measured $J_j/J_{j,0}$ - V_j plot (charged molecules) (see Fig. S3). In addition, if we express FI_1 and FI_2 through their ratio then equation #9 will be as follows:

$$\frac{J_j}{J_{j,0}} = \frac{g_j}{g_{j,0}} \frac{\left(zFV_j/RT\right)\left[\left(FI_1/FI_2\right) - \exp\left(-zFV_j/RT\right)\right]}{\left[1 - \exp\left(-zFV_j/RT\right)\right]\left[FI_1/FI_2 - 1\right]} \quad \#10$$

To estimate P_p, we blocked gap junctional conductance by acidification using CO_2 or long chain alkanols and measured kinetics of FI_2 decay over time. Under blocking conditions, $P_{j,0}=0$ and $V_j=0$, then from the equation #8 it follows that:

$$P_p = \frac{-vol_2\left(\Delta FI_2/\Delta t\right)}{FI_2} \qquad \#11$$

Our data show that P_p can vary in the range of 1.1 to 4 x 10^{-11} cm^3/s. In this equation, it was assumed that: 1) voltage in pipette-2 is equal to voltage in cell-2, and 2) the concentration of dye in pipette-2 is negligible. The latter may not be very true, specifically for pipettes with long tapered tips. For this reason we always made pipettes with tapered tips as short as possible. P_p depends mainly on the size of the open patch at the tip of the pipette, which can vary among experiments. In addition, we assumed that there was no binding of dye to components of the cytoplasm. However, some experiments lasted more than one hour (see Fig. 2), and during this time we can not exclude that a fraction of dye was incorporated into intracellular compartments. Nevertheless, the changes of FI_2 during application of uncoupling agents show that this binding should be much slower than the duration of voltage protocols used, and therefore should not affect substantially evaluation of J_j and P_j. P_p estimates also could be a source for additional errors in evaluation of P_j. However, observed P_j at $V_j=0$ was constant during the experiment (Figures 2, 4, 6). In addition, P_j and J_j approached zero under uncoupling conditions which is an additional indication that P_p estimates were reliable. An estimation of vol_2 was based on the assumption that cells have the shape of a hemisphere. The diameter of a hemisphere was determined by averaging the longest and the shortest diameters of the cell; on average, the volume of examined HeLa cells was ~1800 μm^3. In J_j and P_j evaluations, we neglected dye loss through the non-junctional plasma membrane of cell-2 due to earlier reports showing that dye diffusion through hemichannels or other non-Cx-related mechanisms is at least ~10-fold lower than dye diffusion to the patch-pipette (6).

Single channel flux (J_γ) or permeability (P_γ) can be found by dividing J_j or P_j by the number of functional channels, $N_f=g_j/\gamma$, where γ is the single channel conductance, at any given time, i.e., $J_\gamma=J_j/N_f$ and $P_\gamma=P_j/N_f$. For example, P_γ can be found from equation #7 as follows:

$$P_\gamma = \frac{\gamma\left[\left(vol_2\,\Delta FI_2/\Delta t\right)+P_p\,FI_2\right]\left[1-\exp\left(-zFV_j/RT\right)\right]}{g_j\left(zFV_j/RT\right)\left[FI_1-FI_2\exp\left(-zFV_j/RT\right)\right]} \qquad \#12$$

To increase dye detection sensitivity, which is particularly important in cases where coupling is weak and/or channel permeability is low, time-lapse imaging was performed as follows: the whole visible field was exposed to excitation light with intensity EI_W, followed by focused excitation light with a diameter of ~10 μm with intensity EI_F, and directed only at the dye-recipient cell-2. The latter allowed us to avoid emission light scattering from the dye-donor cell as well as from the dye-filled pipette which can obscure dye transfer to the recipient cell in cases where permeability is low or give the appearance of dye transfer when it is, in fact, absent. Our estimates show that using this approach the sensitivity of dye transfer measurements increases over 100-fold when compared with traditional methods when both cells were exposed to the excitation light. Generally, $EI_W<EI_F$, and we needed to correct for this difference to compare FIs measured in cell-1 and cell-2. In order to do so, at the end of each experiment at time "T", we exposed cell-1 to focused excitation light with intensity, EI_{F*}, to measure fluorescence from cell-1 ($FI_{1(T,F*)}$) slightly below saturation. Soon after (~3 s), we exposed cell-2 with the same intensity of focused excitation light and measured $FI_{2(T,F*)}$ to find the transformation coefficient, $K= FI_{2(T,F*)}/ FI_{1(T,F*)}$. This coefficient allowed us to find a predicted value of fluorescence intensity from cell-2 at time T ($FI_{2(T,W)}$) estimated using EI_W, i.e., $FI_{2(T,W)}=FI_{1(T,W)}$ x K, where $FI_{1(T,W)}$ is fluorescence intensity from cell-1 measured at time T using EI_W. FI_1 shown in all our records was measured using EI_W

(FI_1=$FI_{1(t,W)}$). FI_2 shown in all our records (FI_2=$FI_{2(t,W)}$) was calculated using experimental fluorescence measurements from cell-2 using EI_F ($FI_{2(t,F)}$) as follows:

$$FI_{2(t,W)} = \frac{FI_{2(t,F)}\, FI_{2(T,W)}}{FI_{2(T,F)}} = \frac{FI_{2(t,F)}\, FI_{1(T,W)}\, FI_{2(T,F^*)}}{FI_{2(T,F)}\, FI_{1(T,F^*)}} \qquad \#13$$

where $FI_{2(T,F)}$ is the fluorescence intensity from cell-2 measured at time T using EI_F.

To minimize dye bleaching, we performed time-lapse imaging exposing cells to a low-intensity light for ~0.5 s every 6 s or more. We also used low dye concentrations in the pipette solution, typically 0.1 mM and below, which minimized photo toxicity, but still provided satisfactory fluorescence intensities. We found no difference in fluorescence intensity over time when images were acquired once every 20 s or every 6 s, indicative of minimal dye bleaching. We have used a similar methodology for evaluation of single channel permeability of connexins 30.2, 40, 43 and 45 (6).

Movie S1 shows changes of FIs in cell-1(Cx43-EGFP; in green) and cell-2 (Cx45-CFP; in red) in response to voltage steps of ±25 mV applied to the HeLaCx43-EGFP cell. Fluorescence intensity in cell-2 decayed during positive and increased during negative V_j steps.

Data analysis and statistics. The analysis was performed using SigmaPlot software and averaged data are reported as the means ± SEM.

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