Non-invasive microfluidic gap junction assay

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Gap junctions are protein channels between cells that allow direct electrical and metabolic coupling via the exchange of biomolecules and ions. Their expression, though ubiquitous in most mammalian cell types, is especially important for the proper functioning of cardiac and neuronal systems. Many existing methods for studying gap junction communication suffer from either unquantifiable data or difficulty of use. Here, we measure the extent of dye spread and effective diffusivities through gap junction connected cells using a quantitative microfluidic cell biology platform. After loading dye by hydrodynamic focusing of calcein/AM, dye transfer dynamics into neighboring, unexposed cells can be monitored via timelapse fluorescent microscopy. By using a selective microfluidic dye loading over a confluent layer of cells, we found that high expression of gap junctions in C6 cells transmits calcein across the monolayer with an effective diffusivity of $3.4 \times 10^{-13}$ m$^2$/s, which are highly coupled by Cx43. We also found that the gap junction blocker 18-GA works poorly in the presence of serum even at high concentrations (50 μM); however, it is highly effective down to 2.5 μM in the absence of serum. Furthermore, when the drug is washed out, dye spread resumes rapidly within 1 min for all doses, indicating the drug does not affect transcriptional regulation of connexins in these Cx43+ cells, in contrast to previous studies. This integrated microfluidic platform enables the in situ monitoring of gap junction communication, yielding dynamic information about intercellular molecular transfer and pharmacological inhibition and recovery.

Insight, integration, innovation

Almost all mammalian cells communicate directly with their neighbors by transporting small cytosolic molecules via gap junctions. Here, we utilized an effective microfluidic platform to quantitatively measure transport through gap junctions by hydrodynamically focused calcein, a fluorescent tracer. We observed that high expression of gap junctions in C6 cells transmits calcein across the monolayer with an effective diffusivity of $2.5 \times 10^{-13}$ m$^2$/s. We then compared these cells with cells which have been treated with the gap junction blocker 18-GA, and found that it indeed reduced the lateral dye transfer. In the presence of serum, the effective diffusivity is reduced only slightly, even at high concentrations of the drug (50 μM). However, without serum, the drug is very effective, achieving half maximum effectiveness at low concentrations (0.5 μM). Surprisingly, a washout of the drug after dye loading revealed that the dynamics of recovery is very fast and dose-independent, in contrast to results found for endogenous gap junctions in a previous study. Our method has the advantage of being non-invasive, quantitative, and amenable to in situ drug treatments or washouts that allows the dynamic monitoring of processes with fast dynamics.

Gap junctions are protein channels coupling the cytosolic space of adjacent mammalian cells to allow the direct communication of ions, metabolites, ATP and other small aqueous molecules. The junction is comprised of two subunits, composed of six connexin proteins each, that dock together and form an open channel between cells. There is tremendous diversity in the way they are assembled, as they can be composed of either homogeneous or heterogeneous subunits, resulting in variance in pore sizes or electrochemical regulation (voltage, pH, ionic gating).1

The biophysical properties of a gap junction, such as their conductance or regulation by small molecules and ions, can be readily characterized. Because gap junctional communication allows the fast equilibration of low molecular weight molecules, they are widely known to be involved in the control of cell growth and cell death,2 and coordinate muscle
It is also known that aberrant gap junction physiology, as a result of connexin downregulation, improper trafficking or genetic mutations, can contribute to cancer, cardiac, neurological, auditory, and skin diseases. However, new roles are continually being discovered implying that our understanding of their physiological role is far from complete. Thus, given the dizzying array of gap junctions, the tissues in which they are present, and the molecules that pass through them, it is vital to develop fast, versatile, and high-throughput cell biology platforms for their functional studies across many dimensions of parameter space.

Currently researchers employ either physical injection, scrape-loading, electrophysiological, or electroporation techniques to assess dye transfer. In the microinjection method, a microneedle is used to deliver a bolus of drug or dye into a single cell, while monitoring dye spreading into neighboring cells (Fig. 1a). In scrape loading/dye transfer (SL/DT), a scalpel is used to make an incision into a cell monolayer, allowing the loading of dye into cells whose membrane integrity has been compromised (Fig. 1b). However, these methods suffer many of the following limitations. Single-cell microinjection is very precise and quantitative, but is also very technically challenging and low throughput. A highly skilled technician can reasonably perform only a limited number of microinjections per day. At the other end of the spectrum, scrape-loading dye transfer is fast and accessible, but highly unquantitative due to uneven dye loading and poor cell viability.

In addition, many other methods for studying gap junction communication also exist, including electrophysiological methods, which yield insight into current conduction but not the size limits of junctional permeability, fluorescence recovery or activation, which can be phototoxic or expensive, or radio-labeled metabolic assays, which require cloning steps and are limited in cell type. Electroporation has also been investigated as a way of spatially isolating dye loading, but it is an invasive procedure that can adversely affect cell viability. Each of these approaches is characterized by its own advantages and disadvantages (Table 1), and the most appropriate assay will naturally be application-dependent.

### Table 1 Advantages and limitations of various gap junction assays

<table>
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<tr>
<th>Assay</th>
<th>Advantages</th>
<th>Limitations</th>
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<tr>
<td>Microinjection</td>
<td>Precise and quantitative</td>
<td>Technically challenging and low throughput</td>
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<tr>
<td>Scrape loading/dye transfer</td>
<td>Fast and accessible</td>
<td>Highly unquantitative due to uneven dye loading</td>
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<tr>
<td>Electrophysiological methods</td>
<td>Yields insight into current conduction</td>
<td>Does not reveal size limits of junctional permeability</td>
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<td>Invasive procedure that can adversely affect cell viability</td>
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![Fig. 1](image) Methods for loading dye into a subset of contacting cells. Traditional approaches include microinjection (a) and scrape loading/dye transfer (b). Our approach uses microfluidic dye focusing (c), which can be achieved by focusing a steam of dye between two buffer streams. Calcein dye is used in our application because its acetomethoxy (AM) form is non-fluorescent and membrane permeable. However, once the AM ester is cleaved by intracellular esterases, the molecule becomes fluorescent and can no longer exit the cell (d). This is a useful property for time-lapse monitoring. The photolithographic mask of the device is given in (e), showing the serpentine fluidic resistors at the inputs. A COMSOL simulation shows dye focusing of calcein dye ($D = 2.5 \times 10^{-10}$ m$^2$/s) (f).
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<table>
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<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Microinjection</td>
<td>● precise and quantitative</td>
<td>● low throughput/time consuming</td>
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<td></td>
<td>● instantaneous delivery</td>
<td>● technically challenging</td>
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<tr>
<td>Scrape loading/dye transfer</td>
<td>● fast and simple</td>
<td>● variation in loaded amount</td>
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<td></td>
<td>● many cells analyzed at a time</td>
<td>● invasive/minor cell damage</td>
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<td>gap-FRAP</td>
<td>● many tracers can be used</td>
<td>● invasive/major cell damage</td>
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<td></td>
<td>● quantitative permeability data</td>
<td>● only high density cultures</td>
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<tr>
<td>LAMP</td>
<td>● high spatial precision</td>
<td>● crude and unquantitative</td>
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<td></td>
<td>● high temporal precision (ms)</td>
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<tr>
<td>Electroporation</td>
<td>● only high density cultures</td>
<td></td>
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<tr>
<td>Microfluidic loading/dye transfer</td>
<td>● capability of spatially localizing any membrane-permeable molecule (not just dyes)</td>
<td>● unsuitable for poorly adherent cells</td>
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<tr>
<td></td>
<td>● scalable technology</td>
<td>● requires specialized equipment</td>
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<tr>
<td></td>
<td>● yields quantitative data</td>
<td>● only suitable for monolayer forming cells</td>
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<td></td>
<td>● low reagent consumption</td>
<td>● permeabilization issues, may be difficult to adapt for non-membrane permeable molecules</td>
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Here we demonstrate a non-invasive microfluidic gap junction assay, which has two major advantages: ease-of-use and capacity for quantitation. Previous microfluidic devices for assaying gap junction mediated dye transfer work by vacuum suction trapping two cells adjacent to one another and loading calcein into one of the cells. However, this approach retains the cells in a rounded non-adherent state. In contrast, the assay we have developed integrates adherent cell culture with the subsequent assay, thus allowing the cells ample time to adhere and express functional junctions. In this approach, we can selectively deliver dye by using hydrodynamic focusing of fluids at low Reynolds number ($Re < 1$) into a column of cells within a confluent cell culture chamber, and monitor subsequent dye transfer by fluorescent microscopy. Although, historically hydrodynamic focusing has long been used in flow cytometry for sorting applications, the principle has now garnered attention as a method for precise fluidic delivery of small molecules into cells. Since this approach is entirely non-invasive and highly repeatable, it can potentially be scaled up for high-throughput applications.

**Principle of operation**

The basic principle behind our device is that hydrodynamic focusing of dye allows the spatially targeted delivery of dye into some cells, while leaving their neighbors unperturbed. Thereafter, the subsequent transfer of dye from the targeted cells to their neighbors can be attributed to direct cytosolic transfer (Fig. 1c). For our chosen channel geometries, the Peclet number is on the order of $10^2$, indicating that advective transport dominates over diffusive transport.

In our experiments we used the calcein/acetomethoxy (AM) dye which has many useful properties for our application. Calcein, a normally anionic protein, is not permeable to cell membranes. However, the AM ester modifies the carboxyl groups on the molecule, rendering it non-fluorescent and also non-polar so that the molecules can penetrate the cell membrane. Once inside the cell, however, intracellular esterases cleave off the AM ester, thus uncaging the fluorescence and rendering the dye membrane-impermeable again. Calcein (MW 623) is known to permeate through many types of gap junctions and can be used as a tracer in microinjection experiments.

The properties of calcein/AM are extremely useful for our assay because once a dye molecule is loaded into a cell membrane, it can no longer diffuse back out into the extracellular environment (Fig. 1d). Thus, any spread in fluorescence is restricted to lateral transmission through gap junctions. By timelapse-imaging the cells after dye loading, we can spatio-temporally observe the process of gap-junction mediated dye transfer. Image processing of the data yields biophysical measurements on the rate of dye transfer.

**Experimental methods**
Device fabrication

The microfluidic devices were fabricated by casting polydimethylsiloxane (PDMS) (Dow Corning Sylgard 184) against a negative master. The master was made by photolithographically patterning SU-8 2035 on a silicon wafer. The SU-8 was spincoated to a thickness of 40 μm and exposed using a contact aligner. The masks were designed in AutoCAD and printed on mylar (40 640 DPI, Fine-line Imaging). Wafers were hard baked for 30 min at 150 °C and treated with trichloromethylsilane (Sigma) for 20 min by vapor deposition to facilitate easy removal of PDMS.

PDMS elastomer was mixed with curing agent at a standard ratio of 1 : 10. The polymer was then cast onto the silicon masters and cured at 60 °C for 1 h. After the devices were cut and punched with fluidic inlets, they were treated with an oxygen plasma for 20 s and bonded onto large #2 glass coverslips. The entire device was then bonded onto bottomless polystyrene 96-well plates (Evergreen Scientific), which served as fluid reservoirs for the inlets. A pressure controller with a manifold for 96-well plates was then used to control the pressure of the fluidic inlets (ONIX, CellASIC Inc).

Cell culture

All HeLa and C6 cell lines are maintained in DMEM with 4.5 g/L of glucose, 10% FBS, and 1% penicillin/streptomycin. Cells are passed at 70% confluence using 0.05% trypsin with EDTA (GIBCO).

Device operation

Assembled microfluidic devices were first UV-sterilized for 2 h, then loaded with 10 μg/mL fibronectin and incubated at room temperature for 1 h. Cells are trypsinized (0.25% trypsin with 1 mM EDTA (GIBCO)) and seeded into the culture chambers at high density (10 million cells/mL) from the outlet at 0.1 PSI. The device is then detached from the manifold and the cells are cultured to confluence (about 2–4 days) in a standard incubator (37 °C and 5% CO₂). Gravity-driven flow of the media (400 μl) provided at the inlet reservoirs ensures that the cells are continually perfused with fresh media at a flow rate of approximately 0.2 μL/s, as measured by particle tracking inside the chambers.

Once the cells grow to confluence, all reservoirs are loaded with CO₂-independent medium (GIBCO), supplemented with 1% penicillin/streptomycin (GIBCO), to replace the original DMEM media. An additional 0.05% DMSO is added to the media to control for the additional DMSO when the gap junctional blocker 18α-glycyrrhetinic acid is added. Calcein/AM (Invitrogen) is dissolved in DMSO (Sigma-Aldrich) and diluted into the culture medium to 20 μM. In some experiments, the lipophilic DiI dye is also added to this dye solution to a final concentration of 5 μM. This dye solution is then loaded into the central reservoir. The dye is hydrodynamically focused over the cultured cells by applying high pressure (3.1 PSI) onto the side channels and low pressure (0.1 PSI) into the middle channel. After 5 min of dye loading, the dye stream is stopped and timelapse images are taken at 1 frame per min for 25 min. Forward pressure in the buffering streams is maintained at 0.11 PSI to prevent backflow of dye-laden solution.

For 18α-glycyrrhetinic acid (18α-GA) experiments, cells are treated with the drug in a cell culture incubator for 30 min. All 18α-GA stocks are prepared on the day of the experiment, to a stock concentration of 100 mM. After treatment, the cells are then subjected to the same dye focusing protocol as detailed above. After the dye loading and 25 min timelapse, fresh media is flowed through to wash out the 18α-GA concurrently with continued imaging.

Fluorescence imaging and data analysis

Quantitative fluorescence imaging of cells was performed using a Zeiss AxioObserver Z1 microscope with a Hamamatsu 9100-13 EMCCD camera. Image acquisition was automated using iVision (BioVision Technologies). Five line profiles per data set were chosen manually based on high fluorescence in the dye-loaded cells, to ensure a high signal-to-noise ratio and so that no cell was represented twice.

Results

Device design, simulation, and characterization

The design of the device was constrained by the target width of dye focusing, the minimum pressure that can be applied by our flow controller and the maximum shear that can be reasonably experienced by C6 glioma cells. Previous research has shown that glioma cells can be cultured under shear conditions up to 16 dynes/cm², so we chose a threshold of at most 5 dynes/cm², which is well below the published number. The target width of the dye stream is approximately the width of one adherent cell, in this case: 25 μm. Although subcellular resolution has been achieved, those widths are unnecessary for our application, since we are investigating transport between cells.

We use a pneumatic flow controller to actuate the liquids in our devices. Because the digital resolution of our pneumatic flow controller is 0.1 PSI, or around 0.7 kPa, the effective fluidic resistance of the device must be high in order to supply low flow rates. Using lumped element modeling and assuming a Poiseuille flow profile, we approximate the resistance of a
where \( \eta \) is the viscosity of solution, \( L \) is the length of the channel, \( W \) is the width, and \( h \) is the height. Using this equation and the constraints on flow rate ratios, we designed serpentine channel resistors (49 × 0.1 × 0.05 mm) at each inlet (Fig. 1e), with resistances up to 790 Pa/(\( \mu \text{L} \)/min), resulting in a final flow velocity in the cell culture chamber of approximately 0.07 cm/s (3 dynes/cm\(^2\)). Empirical characterization of the bare devices showed that 25 \( \mu \text{m} \) dye widths can be achieved by applying 0.1 PSI of pressure at the central inlet and 3.1 PSI of pressure at the outer inlet (Fig. 2a–b).

![Dye focusing in bare devices and over confluent cells. Various widths of dye focusing can be achieved with the device by modulating the pressure at the outer inlets with respect to the inner inlet (a). At \( P_i = 0.11 \text{ PSI} \), and \( P_o = 3.1 \text{ PSI} \), dye in bare channels can be focused to a width of 20 \( \mu \text{m} \) (b). Dye focusing over HeLa cells show that calcein/AM and DiI dye can be focused over cells in the center of the channel (c–e). Scale bar: 200 \( \mu \text{m} \).](http://www.rsc.org/delivery/_ArticleLinking/DisplayHTMLArticleforfree...)

With these parameters, finite element simulations also verified hydrodynamic focusing of the central dye stream with sharp boundaries (Fig. 2). Simulations were run assuming incompressible Navier–Stokes flow and a diffusion constant of 2.5 \( \times 10^{-10} \text{ m}^2/\text{s} \) for calcein. One central concern was that a cell monolayer on the bottom surface of the device chamber would introduce topographical variations that would disturb the flow profile significantly. Experimental validation shows that even with HeLa cells cultured in the chambers, the dye is still focused with sharp boundaries to 24 ± 6 \( \mu \text{m} \) (st. dev., \( n = 4 \)) using the...
pressures previously determined (Fig. 2c–d). HeLa cells are reported to have downregulated connexin expression, so after 5 min of dye loading, and 10 min of incubation, the calcein dye is concentrated in the middle of the chamber. Dil dye, a lipophilic membrane dye, was also used as a control to track the spatial localization of the central dye stream (Fig. 2c).

Because Dil is restricted to the membrane and cannot be transferred to adjacent cells, the Dil serves as a control to ensure that the dye stream is not mixing with the buffer streams in the chamber. The Dil fluorescence is more concentrated to the middle of the chamber, indicating that HeLa cells may have some low level of gap junction expression. However, it is more likely that this result reflects the much lower diffusivities of Dil, in solution ($2.5 \times 10^{-11}$ m$^2$/s) or in cell membranes ($3.8 \times 10^{-12}$ m$^2$/s).

Although the presence of cells on the bottom surface of the microfluidic device does not significantly affect sharpness of the dye peak, the cells do disturb the path of the central dye stream, offsetting it from the center of the chamber, or introducing a slight curvature. These disturbances are unavoidable and in subsequent dye transfer experiments, we account for this variation simply by measuring all dye spread distances from the peak, even if the peak deviates from the center.

One major advantage of the microfluidic approach is that dye loading can be accomplished in an entirely non-invasive way, while also yielding dynamic information about the process of dye transfer. In addition, the operation of this device is extremely simple, requiring no syringe pumps or tubing, which can be bulky to transport and can introduce deleterious bubbles into the system. With a pneumatic pressure controller, bubbles rise to the air–liquid interface which is separated from the fluidic inlet by the height of liquid present in the reservoir. Since it is easy to disengage the device from the pressure flow controller, extended periods of cell culture can be done on-chip, without the potential bubble problems that can arise when re-interfacing microfluidic chips with tubing for syringe pumps. Though we use a commercially available pressure controller to actuate the flow inside the microdevice, it is entirely possible to actuate the device by simply applying a vacuum at the outlet, requiring no more than a standard vacuum line and a vacuum controller or vacuum gauge. Additionally, master molds of this feature size can easily be fabricated using simple materials such as shrinky dinks or through a public foundry. Thus, the technique is extremely accessible even to researchers outside the microfluidics community.

**Cx43+ C6 glioma dye transfer**

After validating that dye focusing is intact even over confluent cells, we investigated the dynamic spread of dye in C6 glioma cells stably transfected with connexin43 (Cx43). As is typical of many cancer cell types, the wildtype C6 glioma cells have downregulated the expression of gap junctions. Biochemical characterization of Cx43 protein levels has been performed elsewhere.

We characterized the spread of calcein dye in Cx43+ C6 cells, wildtype C6 cells, and Cx43+ C6 cells which have been treated with the gap junction blocker, 18α-glycyrrhetinic acid. The Cx43+ C6 cells show extensive lateral transfer of calcein dye (Fig. 3b and 4b), compared to the wildtype cells (Fig. 3a and 4a). After treatment with 18α-GA, the same Cx43+ cells are assayed, revealing a drastic reduction in dye spread (Fig. 3c and 4c). The fluorescence profiles across the chamber are given for multiple points along the chamber (Fig. 3) and over time (Fig. 4), yielding dynamic information about gap junctional transfer of dye.
**Fig. 3** Fluorescence intensity profiles across the cell chamber, 30 min after dye loading. Each profile is the column average of the area. Cx43-negative wild-type C6 cells (a) do not exhibit any dye spread, at any point along the chamber. Cx43+ C6 cells (b) show extensive dye transfer, as evidenced by the multiple fluorescent peaks across the chamber. The presence of gap junction blocker 18α-GA inhibits dye transfer (c). Fluctuations in peak intensity can be attributed to fluctuations in the intensity of the illumination source. Scale bar: 100 μm.

**Fig. 4** Fluorescence intensity profiles over time of a given region-of-interest for the different cell types. The profile plotted is a column average of the area (20 pixels width) given in the white dashed box in **Fig. 3**. Wildtype C6 cells (a) show no spreading over 30 min, while Cx43+ cells (b) show a decrease in the fluorescence of the central peak, with a corresponding increase in the fluorescence of neighboring cells. 18α-GA exposure inhibits dye spread (c). Scale bar: 100 μm.

By plotting the average dye spread over time (**Fig. 5a**), we can see that calcein travels approximately 50 μm through the Cx43+ C6 cells in 25 min. We discovered in these experiments that dye transfer is greatly reduced, but not completely abolished, when incubated with 50 μM 18α-GA in serum-containing media. Using the equation for the diffusion length, \( L = \sqrt{4Dt} \), we can calculate an effective diffusivity, plotted in **Fig. 5b**. This calculation yields an effective diffusivity of \( 3.4 \times 10^{-13} \text{ m}^2/\text{s} \) for the Cx43+ cells, translating to a reduction of two orders of magnitude in the diffusivity of calcein in cell cytosol. When gap junctions are blocked by 18α-GA, the diffusivity drops slightly, to approximately \( 1.2 \times 10^{-13} \text{ m}^2/\text{s} \).
Fig. 5 Dye spread and effective diffusivity for Cx43+ C6 cells, wildtype C6 cells, and Cx43+ cells that have been treated with the gap junction blocker 18q-GA. For (a), dye spread is defined as the width of the central fluorescent peak, measured at the point where the fluorescent intensity drops to 36.9% (1/e). Cx43+ C6 cells transfer the dye much further than wildtype C6 cells (error bars denote the standard deviation). Treatment of cells with 18q-GA greatly decreases the dye spread, but does not completely abolish it. In (b), the effective diffusivity is shown, indicating an increase of two orders of magnitude from wildtype C6 cells to Cx43+ cells. Treatment of cells with 18q-GA lowers the effective diffusivity by an order of magnitude. (n = 3 * indicates p < 0.05).

However, we found that when the Cx43+ cells are treated in 18q-GA in the absence of serum, the efficacy of the drug is greatly increased (Fig. 6). Without serum, dye transfer was completely blocked down to 5 μM (Fig. 6e). The observation that serum blocked the activity of the inhibitor corroborates with the studies done on alveolar epithelial cells and on fibroblasts. Both of these studies showed that the presence of serum required concentrations of 18q-GA over 50 μM in order to block gap junction activity.
In the dose response study, we found that the drug is half-maximally effective at 0.5 µM. At this concentration, the pEC50 was $3.8 \times 10^{-14}$ m²/s, about halfway in between the maximum ($3.4 \times 10^{-13}$ m²/s with no drug), and the minimum ($1.78 \times 10^{-15}$ m²/s for 50 µM).

After 25 min of incubation in the drug-containing media, we washed out the drug using serum-containing media and continue the timelapse for another 25 min. These experiments showed that the dynamics of recovery are extremely fast for all doses. Within one minute of the washout, lateral dye spread was detectable for all doses, asymptotically approaching the maximum within 25 min (Fig. 6a–d). The fast dynamics of this recovery for all doses indicates that the primary blocking action of glycyrrhetinic acid is biochemical and not transcriptional in nature, as seen in other cell types at high concentrations (see Discussion).

The low effective diffusivity of calcein provides good evidence that any spread of fluorescence is not a result of lateral calcein diffusion in the extracellular media, which would be much faster. Two other results which verify that the dye spread is indeed gap junction-mediated are shown in Fig. 7. In one set of experiments using Cx43+ C6 cells, we see that a relatively isolated cluster of cells exhibits very low fluorescence compared to other cells at the same distance away from the center. This cluster is connected to the main body of cells through only one cell, thus drastically reducing the number of gap junctional connections that can be made. If the dye could travel by extracellular diffusion, then cellular fluorescence would be independent of membrane contact and dependent only on distance from the midline. Since we see almost no fluorescence in these isolated cells, we confirm that there is no extracellular diffusion. The second result is that membrane-localized DiI dye that is loaded concurrently with the calcein/AM dye shows no spread after 30 minutes of incubation, affirming that the apparent lateral diffusion is not an artifact of diffusive smearing in the laminar streams above the cells. Thus, we conclude that all diffusive spread of dye in our experiments is wholly dependent on direct cytosolic intercellular transfer.
Fig. 7 Further evidence that dye transfer is indeed gap junction mediated. An isolated 3-cell island shows little fluorescence due to a paucity of connections, showing that dye spread is indeed gap junction mediated (a, b, d, e). Additionally, 30-minute endpoint plots (f) of calcein fluorescence (b) and DiI (c) fluorescence in the chamber show that calcein is distributed across the entire chamber whereas the membrane-localized DiI dye is only present in cells in the middle of the chamber. This data set was not included in the analysis for Fig. 5.

Discussion

The quantitative studies done here show that gap junction connected cells allow the transfer of small molecules, such as calcein dye, with an effective diffusivity of $3.4 \times 10^{-13} \text{m}^2/\text{s}$. The gap junction blocker 18α-GA, though clearly inhibiting gap junction communication, does not completely block gap junction communication, resulting in a final effective diffusivity of $1.2 \times 10^{-14} \text{m}^2/\text{s}$.

The results of the washout study contrasts with a dose response study done in alveolar cells, in which high doses (>10 μM) of 18α-GA maintained for 30 min resulted in the disassembly of gap junctional plaques. Removal of the drug in this case did not induce the reassembly of gap junction plaques. However, our results show that Cx43+ C6 cells do not experience long-term reduction in gap junction coupling when treated with 18α-GA. In fact, these cells begin exhibiting dye spread immediately (<1 min) after the drug washout, completely independent of the initial drug dose. The faster dissociation of the drug in our device could be a direct result of the physical shear from continuous flow of fresh media during washout. Future iterations of this device with finer resolution on flow speed could elucidate the shear-dependence of this recovery. Another possible explanation for the discrepancy in results could be that the drug induces transcriptional feedback to downregulate gap junction expression in the other cells, but not in C6 cells. More detailed comparative studies are required to confirm these possible interpretations.

In our study, we restrict the analysis of dye transfer to the x-dimension (Fig. 3a and 4), based on the assumption that dye loading down the central column of cells is relatively even, and that dye transfer only occurs laterally. However, since diffusion occurs in 2D, the study can be easily extended by using more advanced image processing algorithms to detect individual cell boundaries and map the total fluorescence in each cell at each timepoint to accurately measure the flux between cells. Connexin molecules can be GFP-tagged or stained in situ to provide yet another measure of the relative gap junctional density at each cell boundary.

Although our study investigates only the transfer of calcein/AM particles, many other types of molecules can also be potentially loaded by conjugating them to an AM moiety or potentially by encapsulating them in liposomes, which fuse with the cell membrane to deposit their contents inside the cell. The spatially defined delivery of these molecules allows researchers to visually track signal transduction through any lateral cell signaling pathway, such as through connexins, pannexins, adherens junctions, desmosomes, and notch-delta signaling. The microfluidic architecture, as opposed to microinjection for instance, is advantageous in these cases because the continuous flow can aid in decoupling lateral signaling from secreted factor signaling. Additionally this platform makes it simple to perform washout experiments in situ, enabling the continuous monitoring of physiological processes with very fast dynamics. As we continue to develop new experimental methods for dissecting the complex role of cell–cell signaling pathways, we can better understand the basic biology of complex tissues in development and disease.
Conclusions

Gap junction communication is nearly ubiquitous amongst mammalian cells. Their diversity of function and complex role in cell signaling necessitates quantitative methods of measuring gap junction activity. Here, we use microfluidic dye focusing to selectively load fluorescent dye over a confluent layer of cells, so that dye transfer to neighboring cells can be monitored by timelapse fluorescent microscopy. We showed that hydrodynamic focusing can be maintained even over a confluent layer of cells. Using this approach, we found that calcein has an effective diffusivity of \(3.4 \times 10^{-13} \text{ m}^2/\text{s}\) in cells which are highly coupled by Cx43. We also found that the gap junction blocker 18α-GA works poorly in the presence of serum even at high concentrations (50 μM) but is highly effective down to 2.5 μM in the absence of serum. Furthermore, when the drug is washed out, dye spread resumes rapidly within one minute for all doses, indicating the drug does not affect transcriptional regulation of connexins in these Cx43+ cells, in contrast to previous studies. Since the modulations of gap junction in intercellular communication are potential pharmacological targets, our microfluidic platform for gap junction analysis can be useful for fast multiplexed screening and answering fundamental biological questions about the functions of mutational alternations in the connexin genes.

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