Interactions among the cells that make up organs and various tissue types determine the function of living organisms in both the maintenance of healthy homeostasis and the development of disease. In cancer, dysfunctional and co-opted cell communication pathways have been linked to the development of drug resistances, metastasis, and increased recurrence. Numerous interactions have been investigated including cross-talk between stromal and epithelial cell types, localized enrichment of endocrine and growth factor signaling within cell populations, and alterations cell-matrix associations observed in epithelial-mesenchymal transition. Further, experimental evidence and clinical observations have converged in support of the view that the complex, and often highly heterogeneous, tumor microenvironment actively participates in all phases of cancer development. It is this heterogeneity and complexity that demands the development of new experimental techniques that enable identification and manipulation of biological communication pathways starting at a single cell level.

Focal activation of a single cell in a quantitative manner remains a particularly challenging task. The prevailing method of mechanical stimulation does not account for complexities of many communication pathways, and necessitates new methods for precise delivery of messenger molecules to study of various stages of diverse signal cascades. For quantifiable delivery of a broad range of unmodified bioactive molecules to a single cell, nanoparticle- and laser-assisted intracellular delivery techniques are being explored. For example, infrared (IR) laser light can manipulate sub-100 nm lipid vesicles with high refractive index cores, achieved by encapsulating 1 M sucrose, yielding an inducible dipole moment suitable for stable optical trapping. Sub-100 nm metallic particles also efficiently interact with IR optical trapping lasers due to their high polarizability and can be optically injected across lipid membranes to transiently open 100 to 200 nm pores. This process relies on simultaneous plasmonic heating and optical force in the direction of light propagation to push the...
gold nanoparticle through the lipid membrane, leaving a perforation that allows diffusion of extracellular solutes into the cell. Similarly to optical transfection methods, using laser light alone can perforate a cell membrane and allow internalization of nucleic acids. There is, however, no broad method for quantifiable delivery of many types of molecular agents that targets only the single cell of interest while retaining cell viability.

We previously introduced light-sensitive gold-coated liposome for encapsulation and controlled delivery of soluble drugs and contrast agents, as a platform system for therapeutic and diagnostic delivery. Morphology of this composite nanocapsules was elucidated by electron microscopy observations, and their optical properties described by applying the effective medium theory, as summarized elsewhere. Gold-coated liposomes selectively absorb visible to NIR light, providing a photothermal trigger for rapid content release. We have employed gold-coated liposomes for the localized extracellular delivery of active G-protein coupled receptor (GPCRs) agonists. The strong polarizability of the gold shell surrounding the liposome’s lipid membrane enhances optical trapping and facilitates the optical injection of these nanocarriers into cells. We then demonstrated intracellular delivery by optical injection of gold-coated liposomes encapsulating TO-PRO-3, a cell-impermeant fluorescent nuclear dye, while maintaining viability of cells following optical injection.

Here, we introduce the method of focal activation of intercellular communication by optical injection of gold-coated liposomes. We applied a modulated optical trap to propel, or optically inject, 100 nm gold-coated liposomes encapsulating signaling molecules into a single cell. The number of gold-coated liposomes delivered into the single cell was determined by incorporation of a fluorescently-tagged lipid within the liposomes, allowing quantification of the dose of active molecules delivered into the cytosol. As a demonstration of focal activation, we examined intracellular calcium (\(\text{Ca}^{2+}\)) signaling in the ovarian carcinoma cell line OVCAR-3, initiated by precise delivery of inositol trisphosphate (IP3) or adenophostin A (AdA) encapsulated within gold-coated liposomes. IP3 (Figure 1a) is a ubiquitous secondary messenger in GPCR and receptor tyrosine kinase (Rtk) signaling pathways, acting as an agonist of intracellular IP3 receptors (IP3Rs) leading to intracellular Ca\(^{2+}\) release. An analogue of IP3 with greater affinity for IP3Rs, has been shown to exhibit a half maximal effective concentration of 8.3 nM for IP3 subtype 1 versus IP3’s EC\(_{50}\) of 87 nM for the same receptor. Increases in cytosolic \(\text{Ca}^{2+}\) elicted by delivery of liposomal IP3 or AdA were monitored in the single injected cell and neighboring cells using the \(\text{Ca}^{2+}\)-sensitive fluorescent dye Indo-1.22 This application of optical injection represents a new, quantitative method of focal activation of signaling cascades of broad interest in biomedical research, free of many limitations of currently used techniques such as mechanical stimulation or photouncaging.

**RESULTS**

**Spectral Properties of Liposomes.** A total of five liposome formulations were prepared. First, to quantify the delivery of gold-coated liposomes by optical injection, liposomes encapsulating 500 \(\mu\)M IP3 in phosphate buffered saline (PBS) were prepared incorporating 0.05 mol % of rhodamine-B (RhB) conjugated lipid and gold coated (Figure 1c). For initiating Ca\(^{2+}\) signaling, two liposome formulations were prepared encapsulating either 500 \(\mu\)M IP3 or 50 \(\mu\)M AdA in PBS and gold coated (Figure 1d). To assess the effect of injecting gold-coated liposomes into cells without the presence of signaling molecules, a fourth formulation of liposomes was prepared without IP3 or AdA and gold coated (Figure 1e). As the fifth formulation, a portion of the 500 \(\mu\)M IP3-loaded liposomes were left uncoated to serve as the negative control (Figure 1f). Liposomes
had an average diameter of 95–100 nm (number-weighted) prior to gold coating, and 100–105 nm following the gold coating process as determined by dynamic light scattering. Gold-coated liposomes (Figure 1d,e) exhibited peak plasmon resonances around 700 nm (Figure 1g, red line). A similar spectrum was observed for RhB-labeled gold-coated liposomes (Figure 1c) with an additional peak around 570 nm corresponding to RhB absorption. A computationally derived extinction spectrum (Figure 1g, solid blue line) for 100 nm diameter gold-coated liposomes with 2 nm gold shell at a fill factor of 0.76 shows a peak resonance at 700 nm. Similarly calculated polarizability of these gold-coated liposomes (Figure 1g, dashed blue line) is $4.24 \times 10^{-16}$ cm$^3$ at 1064 nm (Figure 1g, vertical line) and is responsible for the gradient force in the highly focused 1064 nm laser beam.$^{16}$ As expected, uncoated liposomes (Figure 1f) exhibited no plasmon resonance (Figure 1g, gray line).

**Quantification of Gold-Coated Liposome Optical Injection.** Under the settings described in Methods, the average power of the optical injection beam in the plane of sample was 10 mW. Optical injection of gold-coated liposomes prepared with 0.05 mol % DPPE-RhB resulted in RhB fluorescence localized within the injected cell, whereas the intensity of fluorescence relates to the duration of optical injection (Figure 2).

Analysis of the intensity of fluorescence originating from a single cell enabled us to establish a quantitative relation between the injection time and the number of cargo-carrying liposomes delivered to the cytoplasm. We calculated the number of injected liposomes by dividing the integrated intensity of fluorescence emitted by an injected cell by that originating from a single liposome (see Methods for details). Figure 3 plots the calculated number of liposomes optically injected into single cells for durations of 10, 30, 45, 60, 90, and 120 s at a constant lipid concentration of 10 mM (approximately $6 \times 10^{10}$ liposomes/μL) and the optical injection laser settings described in Methods. Linear regression analysis of the data ($R^2$ value of 0.992)
indicates that the gold-coated liposomes are injected at the rate of 5.7 s⁻¹ throughout the tested range of injection times, up to 120 s.

To estimate the number of signaling molecules delivered to the cytoplasm in this process, we assumed that the dose of encapsulated molecules delivered to the cell was proportional to the number of injected liposomes, in the manner dependent on the concentration of encapsulated content. For example, at the IP3 concentration of 500 μM, each 100 nm diameter liposome encapsulates approximately 120 IP3 molecules. Ca²⁺ release from the endoplasmic reticulum requires a minimum concentration of 30 nM of the cellular IP3, a level normally activated by upstream agonists to extracellular GPCR or Rtk receptors. To reach the minimum activating concentration of 30 nM IP3, at least 77 liposomes must be delivered into the typical cell volume of 0.5 × 10⁻¹² L. The calibration data obtained using fluorescently tagged gold-coated liposomes (Figure 3) indicates that this threshold is met at approximately 14 s into optical injection.

Optical Injection of Gold-Coated Liposomes Encapsulating Signaling Molecules. We monitored intracellular [Ca²⁺] changes induced by delivery of IP3 or AdA carried by optically injected gold-coated liposomes. The resulting fluctuations in [Ca²⁺]/[Ca²⁺]baseline are determined as the ratio of Indo-1 fluorescence intensity over baseline fluorescence and shown in Figure 4. Column 1 of
Figure 4 shows differential interference contrast (DIC) images of the experimental field of view (FOV) merged with an image of the optical trap focal spot, which indicates the cell selected for optical injection. The increases in the ratio of $[\text{Ca}^{2+}] / [\text{Ca}^{2+}]_{\text{baseline}}$ at 45, 60, 75, and 120 s after initiation of optical injection are shown in Figure 4 columns 2, 3, 4, and 5, respectively. These results are also summarized in Figure 5 as plots of $[\text{Ca}^{2+}] / [\text{Ca}^{2+}]_{\text{baseline}}$ versus time for the optically injected cell and neighboring cells grouped by degrees of separation.
of separation from the single injected cell. All cells in direct contact with the injected cell comprised the first group, all cells in direct contact with the first group comprised the second group, and so on, with each group of cells including those in direct contact with the previous group. An example of this scheme for grouping cell is depicted in Figure 5a.

Optical injection of gold-coated liposomes encapsulating 500 μM IP3 into a single OVCAR-3 cell for 60 s, providing an intracellular concentration of approximately 120 nM, resulted in widespread Ca2⁺ signaling within the FOV beginning at the injected cell and sequentially spreading outward to the neighboring cell groups (Figures 4a–a5 and 5b, and Supporting Information Movie S1). The observed Ca2⁺ signal begins at the injected cell 30 s into optical injection, spreads sequentially by degree of separation to all cells in the experimental FOV, then returns to near baseline at 120 s (60 s after optical injection turned off).

For comparison, we tested AdA, a highly potent agonist of IP3Rs shown to have 10 times greater affinity for IP3Rs than IP3. Optical injection of gold-coated liposomes encapsulating 50 μM AdA into OVCAR-3 cells for 60 s, yielding an estimated intracellular AdA concentration of 12 nM, similarly resulted in widespread and sequential Ca2⁺ signaling (Figures 4b1–b5 and 5c, and Supporting Information Movie S2). Rapid onset of Ca2⁺ signaling at the cell injected with liposomal AdA is observed at around 15 s.

Pretreating the OVCAR-3 cells with 1 mM octanol, a known blocker of gap junctions, effectively confines the observed [Ca2⁺] changes to only the single cell optically injected with gold-coated liposomes encapsulating 500 μM IP3, with no evidence of [Ca2⁺] changes in adjacent cells, even after 120 s of continuous injection delivering an approximate dose of 240 nM IP3 to the cell (Figures 4c1–c5 and 5d, and Supporting Information Movie S3).

Negative control experiments showed negligible changes in cytosolic [Ca2⁺]. The first control, in which gold-coated liposomes without IP3 were injected, showed only a 2% increase in Indo-1 fluorescence intensity over baseline at the injected cell observed at 75 s into injection and no propagation to surrounding cells (Figures 4d1–d5 and Se, and Supporting Information Movie S4). Additional attempts to optically inject IP3-loaded liposomes without the gold coating for 120 s consistently showed no observable Ca2⁺ response (Figures 4e1–e5 and 5f, and Supporting Information Movie S5). Exposing a single cell to the trapping laser alone for up to 120 s in the absence of liposomes also failed to induce observable changes in Indo-1 fluorescence in either the single cell exposed to the optical injection beam or the surrounding cells (Figure 5g, and Supporting Information Movie S6). No Ca2⁺ signal was observed in response to delivery of a subthreshold dose of IP3, as demonstrated by 10 s of optical injection of gold-coated liposomes encapsulating 500 μM IP3 which resulted in a cytosolic IP3 concentration of approximately 20 nM (Figure 5h, and Supporting Information Movie S7).

Propagation of Ca2⁺ Signal. To illustrate how the proposed method of focal activation can aid quantitative analysis of cellular environment, we examined propagation of Ca2⁺ signal. The timing of the Ca2⁺ signal propagation throughout the cells can be used to calculate the velocity of the Ca2⁺ signal or, alternatively, the apparent diffusion coefficient responsible for the spread of observed signal. Figure 6a shows distance of Ca2⁺ signal traveling away from the injected cell plotted against time as grouped by degrees of separation for OVCAR-3 cells activated by IP3- and AdA-encapsulating gold-coated liposomes. The slope of the linear regression fit to these data reveals the velocity of IP3-induced Ca2⁺ signaling as 2.83 μm/s and of AdA-induced Ca2⁺ signaling as 5.20 μm/s.

Alternatively, the wavelike nature of Ca2⁺ signal propagation throughout gap-junction linked cells can
be described using the apparent diffusion coefficient associated with the observed signal. When we plot the mean squared displacement of the outward spreading Ca\(^{2+}\) signal versus time, the apparent diffusion coefficient in two-dimensional space can be estimated from linear regression analysis of the relation:

\[
\langle x^2 \rangle = 4Dt
\]

where \(\langle x^2 \rangle\) is the mean squared displacement, \(D\) is the diffusion coefficient, and \(t\) is time. Figure 6 shows the analysis of the mean squared displacement of IP3- and AdA-induced Ca\(^{2+}\) signaling obtained from the experimental results shown in Figures 4a and 5b for IP3 and Figures 4b and 5c for AdA. These data reveal apparent diffusion coefficients of 0.67 \(\times\) 10\(^{-6}\) and 1.07 \(\times\) 10\(^{-6}\) cm\(^2\)/s for IP3- and AdA-induced intercellular signaling, respectively.

**DISCUSSION**

Here, we demonstrated the versatility, control, and quantitative nature of focal cellular activation by optical injection of gold-coated liposomes. Perhaps most frequently focal activation is achieved by gentle mechanical stimulation of a cell with a micropipet or similar means, which then triggers production of IP3.\(^{26,27}\) While mechanical stimulation is relevant to certain, mechanosensitive cells, the overall diversity of signal transduction pathways demands appropriately versatile methods of focal activation. In particular, arbitrary control of intracellular concentrations of various messenger molecules may enable investigation of specific stages of many signal transduction pathways of interest to biomedical researchers. Local delivery of molecular agents to single cells can be achieved by microinjection,\(^{28,29}\) optoporation,\(^{30,31}\) and photolysis of chemically modified agonists or photouncaging.\(^{32–35}\) Each of these methods carries inherent technical limitations. For example, while microinjection allows quantitative delivery of molecules to a single cell in small volumes, micropipet contact can easily result in cell damage and unintentional mechanical stimulation of cell signaling, masking specific cellular responses to agonists. Optoporation techniques, either enabled by light absorbing membrane dyes or femtosecond lasers, transiently open a cell’s membrane for passive diffusion of extracellular molecules into that cell. Smallest pore sizes are determined by the diffraction limited laser spot size, typically hundreds of nanometers, and have been shown to persist around 20 s.\(^{36}\) With these and other membrane perforation techniques,\(^{9}\) the dose of active molecules and the rate of delivery is dependent on large extracellular concentrations and, generally unknown, kinetics of that process. Realizations of such experimental techniques in the context of cell signaling are further complicated by the possible influx of Ca\(^{2+}\) or signaling agents from the extracellular space, and release of ATP and other cellular components from the cytosol.

Alternatively, photouncaging techniques provide high temporal and spatial resolutions of delivery; however, they rely on chemical compounds not designed to cross the cellular membrane and therefore require preloading in the cell, possibly by microinjection or optoporation. Molecules for photouncaging techniques must be chemically modifiable, so types of signaling molecules amenable to this technique are limited. Given the complexity of multistep syntheses, applicability of photouncaging in biomedical laboratories is typically limited to compounds available commercially.\(^{35}\)

The optical injection of gold-coated liposomes process that we report here addresses many of these challenges. In this technique, the focal spot of the optical trap is purposefully placed within the targeted cell. In this manner, we create an optical force field capable of accelerating nanoparticles in the manner dependent on their polarizability. Gold-coated liposomes diffuse freely in the extracellular space and, once they encounter the optical force field, are propelled toward the focal point of the trap. Moving toward the focal point, they are exposed to increasing densities of electromagnetic field which in turn deposit increasing amount of heat, eventually resulting in thermal destabilization and content release (Figure 7a). Notably, content release in this setting does not require any specific activation by the operator of the optical trap, rather it is an inevitable consequence of the gold-coated liposomes propelled to its own demise.

The gold-coated liposomes used for this experimental work were specifically made with peak plasmon resonance around 700 nm, well below the 1064 nm wavelength of trapping beam, to reduce absorption of light by these liposomes (40% of its value at maximum,
Figure 1g) and subsequent thermal damage and premature leakage of content. However, at 1064 nm these gold-coated liposomes retain significant polarizability (80% of its maximal value, Figure 1g), resulting in greater optical forces and ability to steer the liposomes toward the cell.

Our proposed mechanism of delivery into the cell’s cytosol involves two events, breaching the cell membrane followed by disruption of the gold-coated liposomes structural integrity. Experimental separation of these two events remains challenging, although we show that photoporation is not involved there. Exposing a single cell to the trapping laser for 2 min without gold-coated liposomes in the extracellular medium resulted in no detectable decrease in Indo-1 fluorescence (Figure 5g), indicating that the integrity of the cellular membrane was maintained. A perforated cell would rapidly lose Indo-1 molecules to the extracellular medium. This agrees with our earlier optical injection work that demonstrated laser trap does not compromise integrity of the cell membrane.16

Liposomal encapsulation is applicable to a broad class of water-soluble factors, including exogenous probes such as TO-PRO-3, previously demonstrated by our group,16 or second messengers, exemplified here using IP3 and its analogue AdA. We selected activation of IP3R for this demonstration as this represents one of many biological processes deregulated in cancer.37 Different research groups have shown that IP3R is the target of proto-oncogenes, such as protein kinase B,38 and tumor suppressors, for example pro-myelocytic leukemia protein.39 Precise intracellular delivery of IP3 and its potent analogues described here may aid in studying deregulated properties of cancer cells and mapping altered signaling networks. Liposomal encapsulation is performed with no chemical modification, leaving no residual background activity of the molecule, which is a frequent problem accompanying caging of biological molecules. Delivery and release can be performed in a quantitative manner, and it is possible to adjust gold-coated liposome or encapsulated solute concentrations to fine-tune the rate and quantity of delivered compound in a very broad range. At the 10 mM lipid and 500 μM IP3 concentrations used here, we typically required 30 s of injection to activate Ca2+ signaling. Conceivably, liposomes can be prepared at 60 mM lipid and 120 mM IP3 concentration (the maximum IP3 solubility in PBS). At this upper limit, an above-threshold intracellular IP3 concentration of 95 nM can be achieved within 30 ms of injection. For attenuated and more precise delivery, gold-coated liposomes prepared encapsulating 4 μM IP3 (resulting in approximately 1 IP3 molecule per liposome) diluted to an effective 100 μM lipid concentration can be used to count and deliver single IP3 molecules into the cell. In this model, we make the assumption that the number of liposomes optically injected deliver the entire payload of encapsulated IP3, however, in the case that there is premature leakage of encapsulated IP3 prior to cellular entry, the amount delivered may be less.

Focal activation of cells by optical injection of gold-coated liposomes enables quantitative analysis of the resulting intracellular Ca2+ wave. As demonstrated in Figure 6a, plotting the time course of the wave propagation reveals signaling velocities of 2.83 and 5.20 μm/s in response to IP3 and AdA injections, respectively. For comparison, intercellular Ca2+ waves are typically reported to travel between 10 and 20 μm/s,7 and 8 μm/s for thymic epithelial cells.25 We also demonstrated that this method can be used to measure the apparent diffusion coefficient of intracellular Ca2+ signaling. We calculated this to be 0.67 × 10−6 and 1.07 × 10−6 cm2/s in response to delivery of IP3 and AdA, respectively, values that are within the range of published diffusion coefficients for intracellular Ca2+, 0.13 × 10−6 cm2/s and cytosolic IP3, 2.8 × 10−6 cm2/s (Figure 6b).7

Optical injection of gold-coated liposomes described here combines both controlled delivery and release of molecules of interest. In comparison, recent works from several laboratories explain how plasmon resonant nanoparticles illuminated with laser light can initiate intracellular release of a drug, nucleic acid, or fluorescent probes once associated carriers are delivered to cells by molecular targeting or nonspecific endocytosis.40–42 Similarly, efficient photothermal conversion in liposome-embedded indocyanine green, a near-infrared molecular dye, can be used to trigger intracellular release of the contents of such liposomes taken by a cell via endocytosis.43 However, all these methods require some other, efficient mechanism of cellular entry, either nonspecific or based on activity of a specific molecular marker triggering cellular entry. Moreover, none of these methods enable addressing individual cells in their microenvironment. Free of these limitations, optical injection of gold-coated liposomes is well suited for studying phenotypic heterogeneity of cells, independent of varying patterns of expression of molecular markers in the cell membrane. As cell populations actually can behave in a functional heterogeneous manner,44,45 the ability to interrogate a single cell with bioactive agents and subsequently monitor the response facilitates a better understanding of phenotypic variations within cell populations.

Quantitative focal activation can also find applications in interrogating cell communication pathways in multicompartmental models of the tumor microenvironment, such as co-cultured or 3D models. In one illustrative example, optical injection of gold-coated liposomes encapsulating 500 μM IP3 was applied to interrogate Ca2+ signaling in a co-cultured model of OVCAR-3 ovarian carcinoma cells in direct contact with supportive fibroblasts (Figure 8, and Supporting Information Movie S8). Propagation of Ca2+ signaling was...
confined to the island of OVCAR-3 cells and did not spread into the surround fibroblasts, indicating gap-junction coupling between OVCAR-3 cells but not between OVCAR-3 and fibroblast cell types, as expected due to limited presence of direct communication pathways between these physiologically separate cell types.

CONCLUSIONS

We have demonstrated quantitative focal activation of individual cells by the use of optical manipulation to gold-coated liposomes. Specifically, we demonstrated how this quantitative focal activation may be used to determine parameters of cellular communication. Because encapsulated molecules require no chemical modification, we believe this method of quantitative intracellular delivery can be applied universally to any water-soluble molecules, while maintaining diffraction limited spatial resolution in combination with doses (zeptomoles) and volumes (attoliters) unattainable by traditional microinjection and photouncaging techniques. Optical injection of gold-coated liposomes may enable the study of single cell dose-response curves for delivery of therapeutics and potentially elucidate functional heterogeneity of cells within a normal tissue or a tumor microenvironment. A better understanding of complex signaling networks beginning at a single cell level, facilitated by the novel experimental method of optical injection of gold-coated liposomes, could lead to improved therapies and diagnostics.

METHODS

Preparation of Gold-Coated Liposomes. Five formulations of thermosensitive liposomes were prepared from synthetic lipids similar to a composition previously demonstrated for temperature-sensitive content release. The general formulation consisted of dipalmitoylphosphatidylcholine (DPPC), monopalmitoylphosphatidylcholine (MPPC), and dipalmitoylphosphatidylethanolamine-N-(methoxy(polyethylene glycol)-2000) (DPPE-PEG2000) (Avanti Polar Lipids; Alabaster, AL) at a 90:10:4 molar ratio. The lipids were dissolved in chloroform, dried under nitrogen, and remaining solvent was removed under vacuum. Dried lipids were combined with PBS that included IP3 molecules to reach a lipid concentration of 60 mM.

Liposomes were prepared by freeze-thaw cycling followed by extrusion through 100 nm porous polycarbonate membranes. Unencapsulated IP3 was removed via three rounds of dialysis using 100 000 molecular weight cutoff cellulose membranes (Spectrum Laboratories; Rancho Dominguez, CA). To form the plasmon resonant gold shell on the surface of the IP3-loaded liposomes, 18 μL of 100 mM gold chloride was reduced by 27 μL of 500 mM ascorbic acid in a 1 mL liposomal suspension at a 10 mM lipid concentration. This process is similar to the technique we previously reported.11

A portion of the IP3-loaded liposomes was left uncoated for control experiments.

The first formulation of liposomes included 0.05 mol % dipalmitoylphosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (DPPE-RhB) (Avanti Polar Lipids) with the other lipid components, encapsulating 500 μM IP3 in PBS, and was gold coated. The second formulation of liposomes was prepared the same as the first formulation, but without DPPE-RhB. The third formulation was prepared similarly to the second, however, encapsulating 50 μM A2A in PBS instead of IP3. The fourth formulation was prepared the same as the second and third, however, without including signaling molecules in the PBS. The fifth and final formulation of liposomes was aliquoted from the second formulation and was not gold coated.

The presence of the plasmon resonant structure was immediately evident by a change in suspension color, and the spectral position of peak plasmon resonance was determined via absorption spectra obtained on a Cary 5 spectrophotometer. Liposome size distributions were determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, U.K.).

Cell Culture. The majority of the experiments described here used OVCAR-3 cells (ATCC, Manassas, VA), a human epithelial ovarian cancer cell line. OVCAR-3 cells were subcultured under standard cell culture conditions in RPMI medium supplemented with 20% fetal bovine serum, 0.01 mg/mL bovine insulin, and
Immediately following optical injection, excess RhB-tagged liposomes at a 10 mM lipid concentration in PBS was exchanged for the PBS in the cell chamber and optically injected into a single OVCAR-3 cell for 10, 30, 45, 60, 90, or 120 s. Immediately following optical injection, excess RhB-tagged liposomes were gently removed with two PBS washes before
these was assigned as the expected fluorescent intensity contribution from a single injected liposome.

**Optical Injection of IP3 and A2A.** For optical injection experiments, we used gold-coated liposomes containing IP3 or A2A for the purpose of eliciting a Ca\(^{2+}\) response. Coverslips with OVCAR-3 cells grown to confluency were washed in PBS and incubated with 8 \(\mu\)M Indo-1 AM (Invitrogen, Grand Island, NY), a Ca\(^{2+}\) sensitive dye, in RPMI medium for 25 min. Indo-1 exhibits peak excitation at 338 nm and has two distinct emission peaks, at 400 and 475 nm, that increase or decrease, respectively, with increasing Ca\(^{2+}\). The 400 nm Indo-1 emission peak was observed for its greater sensitivity to changes in Ca\(^{2+}\) as compared to the 485 nm emission peak. Following incubation with Indo-1, the coverslips were washed twice in PBS and assembled into the cell chamber, described above, containing 5 \(\mu\)L suspensions of gold-coated liposomes, encapsulating 500 \(\mu\)M IP3, 50 \(\mu\)M A2A, or just PBS, at a 10 mM lipid concentration.

Similar to above, the cell to be optically injected was randomly selected from the FOV under DIC, and the position of the laser’s focal spot in the subsequent Indo-1 400 nm emission, the following optical injection experiments. The experimental conditions as described above, but instead of suspensions of gold-coated liposomes, either a suspension of uncoated liposomes or coverslips were washed twice with PBS and loaded into the cell chamber with the cells, and exposed to the trapping laser for 120 s.

To temporally block gap junctions between the OVCAR-3 cells, 1 mM octanol in 1 mL PBS was added to the coverslips with cells for 10 min immediately following Indo-1 incubation. Coverslips were washed twice with PBS and loaded into the cell chamber with gold-coated liposomes encapsulating IP3 for optical injection under the same protocol as described above.

Images acquired during optical injection experiments were saved as 16-bit TIFFs for DIC images and multipage TIFFs for Indo-1 fluorescence images and processed using ImageJ software. To determine changes in Indo-1 fluorescence intensity over time in individual cells, the footprint covered by each cell in the experimental FOV was manually outlined and the average fluorescence intensity confined within each cell was calculated for the duration of the experimental image sequence. Average fluorescence intensity was determined for every cell in the FOV beginning with the injected cell, followed by the first group of neighboring cells in direct contact with the injected cells, then the second group of neighboring cells in direct contact with the first group of cells, and so on. Typical experimental FOVs contained between three and six cell groups spanning outward from the injected cell.

To calculate the relative changes in the ratio of \([\text{Ca}^{2+}]_\text{Indo-1}\)/\([\text{Ca}^{2+}]_\text{Baseline}\) for the duration of the experimental sequence, the average fluorescence intensity for each cell was divided by the average baseline fluorescence intensity, generated from the first 30 s of baseline Indo-1 fluorescence images.

To generate images showing changes in \([\text{Ca}^{2+}]_\text{Indo-1}\), the first 30 s of baseline Indo-1 fluorescence images were averaged to form a single baseline image. This baseline image was then subtracted from subsequent Indo-1 fluorescence images to highlight cells that were exhibiting increases in cytosolic \([\text{Ca}^{2+}]_\text{Indo-1}\).

**Conflict of Interest:** The authors declare no competing financial interest.

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**Supporting Information Available:** Supporting Information Movies S1–S8 as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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