Lab on a Chip

TECHNICAL INNOVATION

View Article Online
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Cite this: *Lab Chip*, 2016, **16**, 2434

Temporally resolved direct delivery of second messengers into cells using nanostraws†

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Received 7th April 2016,
Accepted 1st June 2016

DOI: 10.1039/c6lc00463f

www.rsc.org/loc

Second messengers are biomolecules with the critical role of conveying information to intracellular targets. They are typically membrane-impermeable and only enter cells through tightly regulated transporters. Current methods for manipulating second messengers in cells require preparation of modified cell lines or significant disruptions in cell function, especially at the cell membrane. Here we demonstrate that 100 nm diameter 'nanostraws' penetrate the cell membrane to directly modulate second messenger concentrations within cells. Nanostraws are hollow vertical nanowires that provide a fluidic conduit into cells to allow time-resolved delivery of the signaling ion Ca^{2+} without chemical permeabilization or genetic modification, minimizing cell perturbation. By integrating the nanostraw platform into a microfluidic device, we demonstrate coordinated delivery of Ca^{2+} ions into hundreds of cells at the time scale of several seconds with the ability to deliver complex signal patterns, such as oscillations over time. The diffusive nature of nanostraw delivery gives the platform unique versatility, opening the possibility for time-resolved delivery of any freely diffusing molecules.

Introduction

Manipulating biological signaling cascades is an essential tool in understanding disease,[1,2] drug development,[3] and fundamental biology.[4–6] Signaling cascades are initiated by extracellular molecules binding to receptor proteins at the cell membrane,[7] followed by rapid release of soluble secondary messengers. These bind to downstream intracellular proteins leading to changes in gene expression, protein translation, and phenotypic outcomes[8] and are thus critical to biotechnology and pharmaceutical development. Significant research has gone into finding biological or chemical means to manipulate signaling cascades due to the difficulty of delivering these species across the cell membrane.[9,10] However, current methods, including gene modification,[11,12] optogenetics,[13] chemical genetics,[14–16] and molecular cages,[17–19] are limited due to off-target effects, difficulty in isolating a single control gene, poor temporal resolution, limited cargos, and photodamage.

With the recent advent of cell-penetrating nanostructures,[20–23] it may now be possible to transport the components of biological signaling cascades directly through the cell membrane, obviating the need for gene alteration or chemical perturbations. These cell penetrating structures have been shown to change certain cell behaviors and morphology without preventing migration or proliferation or altering transcription levels.[27–29] Here we demonstrate for the first time that cell-penetrating "nanostraws" provide direct, temporal control of a membrane-impermeant second messenger, Ca^{2+}. The platform consists of 100 nm diameter hollow nanotubes that penetrate through the cell membrane with minimal perturbation, providing a long-term conduit into the cell cytoplasm (Fig. 1). Unlike solid-core nanowires coated with molecular delivery agents, the nanostraws allow temporal modulation of the materials through a microfluidically coupled backplane.[23,30–32] This technique is highly advantageous in that it provides delivery of arbitrary second messengers, small molecules, or proteins using the same platform.

For highly dynamic second messengers, it is important to show sharp on/off signal contrast and deliver material on the time scale of seconds.[24,25] Microchips have demonstrated promise to this end by incorporating microfluidic valves and switching,[26] while nanoscale methods reduce sample volumes to further improve temporal switching resolution and delivery speed. However, the temporal requirement restricts the use of certain nanoscale techniques such as electroporation[38] and nanoparticles,[39] which are better suited to deliver biomolecules such as nucleic acids and proteins. On the

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† Electronic supplementary information (ESI) available: Movies of one-time Ca^{2+} delivery and oscillating delivery. See DOI: 10.1039/c6lc00463f

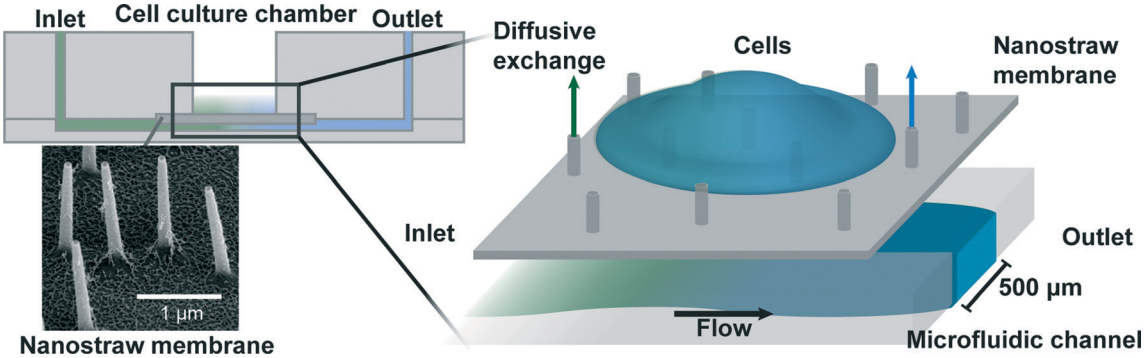

Fig. 1 Nanostraw microfluidic device schematic. Delivery solutions flow through the channel and diffuse into the cell culture chamber (5 mm diameter) through the nanostraws (not drawn to scale). If a nanostraw penetrates into a cell, the delivery solution can enter the cell. Microfluidic access enables delivery of soluble molecules to many cells positioned over the delivery channel and allows the solutions to be quickly exchanged.

other hand, Ca^{2+} ions are one of the most physiologically significant second messengers,[33,34] with high diffusivity[35] and fluorescent indicators that are robust and well-characterized.[36,37] Using the nanostraw platform, we deliver Ca^{2+} directly into cells and further control the delivery to oscillate temporally, as indicated by two different reporters. Ultimately, this technique could be used to address any stage of a signaling network where the messenger is soluble and freely diffusing.

Experimental

Nanostraw membrane fabrication

Nanostraws were fabricated as previously described[23,30] using 100 nm pore diameter track etched polycarbonate membranes as the template at a pore density of 3×10^7 pores per cm^2. Membrane templates for nanostraws were custom-ordered from GVS (formerly Maine Manufacturing, Sanford, ME). ALD was performed on the Savannah platform (Cambridge Nanotech, Waltham, MA). A conformal 15 nm coating of Al_2O_3 was applied to all surfaces of the template by performing atomic layer deposition (ALD). Alumina ALD consists of alternating cycles of trimethylaluminum (TMA) and water (H_2O) exposure with pulse lengths of 0.015 s of precursor, 30 s of exposure, and 60 s of purging to ensure that the precursors coated the entire length of the nanoporous membrane with a 15 nm coating of Al_2O_3. Nanostraws were created by etching the upper alumina surface using a PlasmaQuest etcher and BCl_3 and Cl_2 plasma and removing some of the polycarbonate template to expose the nanostraws with oxygen plasma treatment using a SPI Plasma Prep III Solid State. Typical nanostraw dimensions were 100 nm in diameter and 1.5 μm in height.

Microfluidic device assembly

The nanostraw device consisted of a poly-dimethyl-siloxane elastomer (PDMS) delivery channel, 100 μm deep and 0.5 mm wide, that contained the delivery solution, running below the nanostraw membrane (Fig. 1). A second PDMS piece containing the culture well and cells sealed above the nanostraw membrane, so that the cargo diffused through the nanostraws to enter the culture chamber. The top PDMS piece also contained one or two inlet ports and an outlet for access and temporal control of the delivery channel contents. After assembling PDMS pieces, devices were sterilized in oxygen plasma.

Cell culture, stable line preparation and calcium indicator loading

Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 U mL^{-1} penicillin/streptomycin under standard culture conditions. Prior to cell plating, nanostraw microfluidic devices were coated with 150 μM poly-L-lysine or poly-ornithine overnight (>8 h) at 37 °C. CHO cells were trypsinized and plated at 10 000 cells per device for acute delivery and 100 000 cells per device for oscillating delivery. Delivery was recorded by flowing solutions through the inlet ports and observing cells loaded with one of two reporters: GCaMP6s, a genetically encoded single fluorophore calcium sensor protein,[40] or Fluo-4 AM, a fluorescent chemical calcium indicator.[41]

A eukaryotic expression plasmid for GCaMP6s was generated by subcloning GCaMP6s (Addgene) into a lentiviral vector with an ubiquitin promoter (FUGWm). The resulting construct (FU-GCaMP6s-Wm) was sequenced to verify the cloning and final sequence. CHO cells were transfected with the GCaMP6s plasmid using Lipofectamine 2000. After two weeks of selection using 500 μg mL^{-1} G418 supplemented media, GCaMP6s CHO cells were suspended in culture media (~2 mM Ca^{2+}), and 1 μM ionomycin was added to the solution to increase the fluorescence of GCaMP6s-positive CHO cells for cell sorting. GCaMP6s CHO cells were then sorted by fluorescence activated cell sorting (FACS) for fluorescence three orders of magnitude above background and were propagated for Ca^{2+} delivery experiments. These cells were used for acute delivery studies due to their consistent indicator levels.

Fluo-4 AM was suspended in DMSO and 20% Pluronic F127, and CHO cells were loaded with a final concentration of 1 μM Fluo-4 AM in DMEM and 0.02% Pluronic F-127 (0.1% DMSO) for 30 min prior to Ca^{2+} delivery and imaging. Fluo-4 is more readily available and does not require prior cell modification as GCaMP6s does, but without the benefit of prior cell sorting, the indicator levels are more variable making it more suitable for slower, oscillatory signal measurement. All cell culture and fluorescence dye reagents were obtained from Invitrogen (Carlsbad, CA).

Microfluidic pumping and imaging

To perform Ca^{2+} delivery, cells were plated into microfluidic devices for 24 h prior to imaging. Tyrode's solution (119 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, 30 mM glucose) was used as the basis for delivery and imaging solutions, and supplemented with 300 mM or 100 mM Ca^{2+} for acute and oscillating delivery, respectively. Two syringe pumps (New Era Pump Systems, NE-1000) were attached to the inlet ports with Tygon tubing, and Ca^{2+} solutions were pumped into the inlets at a rate of 0.1 mL min^{-1}, which resulted in solution exchange in the channel volume within 1 ms. For oscillating delivery, a syringe pump injected Ca^{2+} solution for 30 s and turned off, upon which the second syringe pump began injecting 100 mM EGTA solution until the signal returned to near basal levels to complete each oscillation.

Both acute delivery (GCaMP6s reporter) and oscillating delivery (Fluo-4 indicator) were imaged under the same conditions. Wide-field epifluorescence imaging was performed using an Axiovert 200 M inverted microscope (Zeiss) with a 10×/0.3 NA objective (Zeiss, EC Plan-Neofluar) using a Rolera EM-C^2 EMCCD camera with an image capture rate of 1 frame per s. Fluorescence filter set 10 (Zeiss, 450-490 Excitation, 515-565 Emission) was used with an X-Cite 120Q excitation light source. Images were processed by and analyzed using ImageJ. For acute delivery, total cell counts were determined by counting fluorescent cells after long term Ca^{2+} exposure (Movies S1 and S2†). Following background subtraction and alignment, average cell intensity was measured and plotted.

Results and discussion

To demonstrate one-time Ca^{2+} delivery into cells, GCaMP6s CHO cells were plated at low density onto the nanostraw device. Previous studies have shown that cells cultured on nanostraws remain viable and minimally perturbed for an extended period of time.[23,30] In this study, the cells remained viable and responsive to the Ca^{2+} pulse 24 hours after plating (Fig. 2a), and the fluorescence response was imaged for cells cultured on nanostraw membranes and flat membranes (Fig. 2b). The initial Ca^{2+} response within the first 5 s shows concerted and near-universal cellular delivery of Ca^{2+} on nanostraws compared to the infrequent response rate observed in cells on flat membranes (Fig. 2c). The initial rise in intracellular Ca^{2+} reported by the GCaMP6s indicator was observed within 1 s after delivery on nanostraws (N = 138). In the first 5 s, ~90% of nanostraw-delivered cells exhibited a response ($\Delta F/F > 0.5$), while <5% of cells on the flat membrane exhibited an equivalent response (N = 217). This difference indicates that nanostraws facilitated a rapid and concerted flux of Ca^{2+} into the cytoplasm of cells.

When the Ca^{2+} signal was sustained in the delivery channel for longer than 5 s, the cells on the nanostraws maintained a near-constant level of fluorescence with some

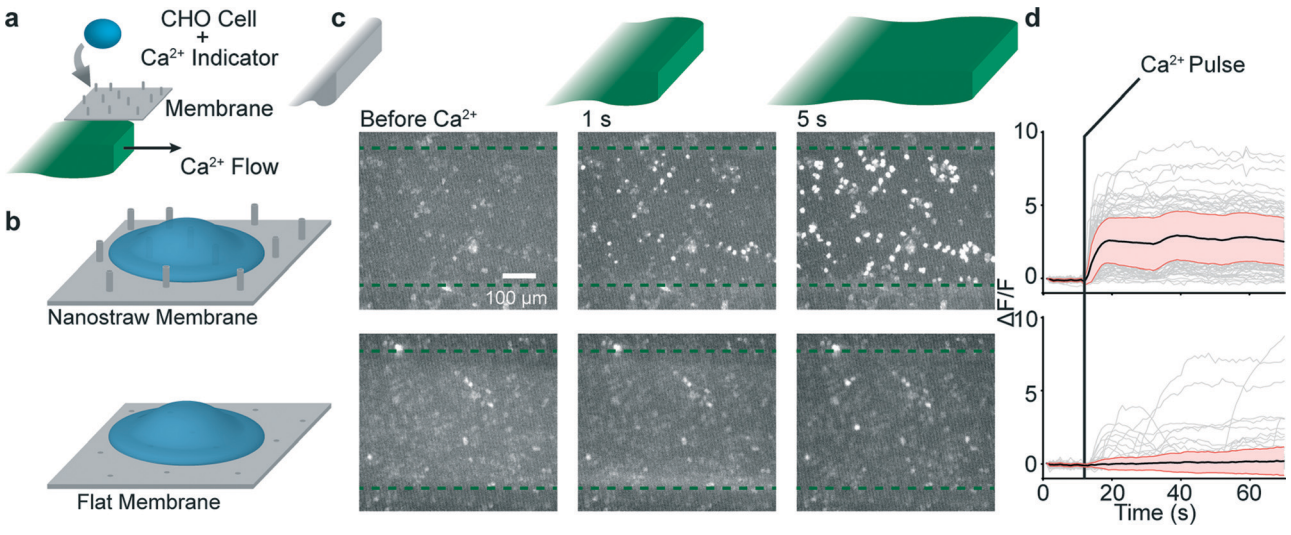

Fig. 2 One-time Ca^{2+} delivery using nanostraws. (a) CHO cells with Ca^{2+} indicators were plated onto nanostraw devices under two conditions (b): cells on nanostraws and cells on a flat membrane with the same pore density without nanostraws. (c) For one-time delivery, a 300 mM Ca^{2+} pulse was added, and cells were imaged before delivery and 1 s and 5 s after delivery. (d) A plot of the change in fluorescence over initial fluorescence ($\Delta F/F$) observed in each cell (gray lines) and the average (black line with standard deviation) demonstrates that nanostraws facilitate second messenger delivery while the flat membrane does not.

minor variation (Fig. 2d, Movie S1 in the ESI,† 20× speed). In contrast, as the Ca^{2+} signal was sustained on the flat membrane without nanostraws, some additional delivery events were observed, but they were rare and sporadic for up to 90 s, after which fluorescence was observed across all cells (Fig. 2d, Movie S2† 20× speed). While a coordinated Ca^{2+} response was eventually observed in both conditions, the flat membrane condition demonstrates the time required for cells to take up Ca^{2+} when it is being pumped into extracellular solution instead of directly into cells through nanostraws.

In addition to detecting one-time Ca^{2+} delivery using nanostraws, a temporally oscillating Ca^{2+} signal was presented to densely plated cells to demonstrate large scale dynamic control over second messenger delivery by leveraging the rapid diffusive exchange through nanostraws. The oscillatory signal consists of alternating flows of 100 mM of either Ca^{2+} or the chelating agent EGTA in Tyrode's solution presented to Fluo-4 loaded cells (Fig. 3a). In response to this signal pattern, oscillating Ca^{2+} reporter fluorescence was observed for 3–4 cycles (Fig. 3b and c, Movie S3,† 225× speed). These results demonstrate that the nanostraws provide a continuous pipeline for delivery into cells, whose contents can be rapidly exchanged. Cell fluorescence during the EGTA phase of the oscillatory signal is reduced to levels comparable to baseline fluorescence prior to Ca^{2+} delivery. The EGTA may be actively diffusing into cells to chelate Ca^{2+} or its presence in the delivery channel may simply reverse the concentration gradient to induce Ca^{2+} to diffuse out of the cell after nanostraw delivery.

We used lower levels of Ca^{2+} for oscillating signal delivery than for one-time delivery to match the solubility limit of EGTA and to ensure that the stoichiometry of Ca^{2+} to chelator binding was not rate-limiting. The lower concentration produced smaller reporter signals and also resulted in slower response times, as expected from diffusion dynamics. Cell-to-cell differences were also more pronounced using Fluo-4 with the oscillating signal compared to one-time delivery of elevated Ca^{2+} to GCaMP6s cells, likely due to a combination of inherent nanostraw delivery variation[30] and varying Fluo-4 AM indicator loading levels compared to more uniform GCaMP6s indicator levels in sorted cells. While perfusion speeds were fast enough to exchange the entire volume in the delivery channel in <1 s, edge effects were expected and observed, perhaps resulting in non-uniform flow and further slowing the response time after several cycles.

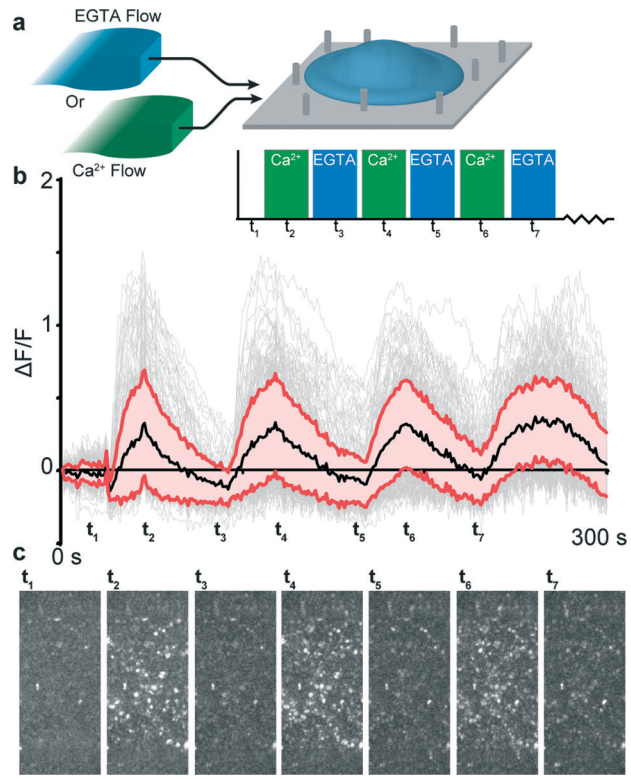

Fig. 3 Oscillating Ca^{2+} delivery using nanostraws. (a) CHO cells with Ca^{2+} indicators were plated on nanostraw devices and alternating flows of 100 mM Ca^{2+} and EGTA were added to the delivery channel. (b) Individual $\Delta F/F$ signals per cell and the average were plotted, demonstrating that nanostraws can facilitate the delivery of oscillating cycles of second messengers into cells. (c) Images at timepoints (t_{1-7}) corresponding to changes in delivery solution show three successful cycles of Ca^{2+}/EGTA before the cells begin to lose their coordinated response to delivery.

Conclusions

Here we have demonstrated the direct manipulation of the second messenger Ca^{2+} without requiring the use of genetic manipulation, chemical carriers, or cell permeabilizers. Ca^{2+} is one of the most ubiquitous and important second messengers, and while a number of effective Ca^{2+} regulating tools are available, demonstrating Ca^{2+} manipulation inside cells is a powerful proof of concept towards the study of lesser known second messengers for which alternative techniques such as photo-uncaging or optogenetics are not an option. On the nanostraws, the cells remained viable after 24 hours of culture and responsive to the Ca^{2+} signal, be it a single acute pulse or an oscillatory sequence. Previously, nanotechnology has been applied to increase the efficiency of techniques such as electroporation.[32,38] The nanostraws provide a similar scaling effect to microinjection to apply a high degree of cell access and delivery to large numbers of cells.[30]

Future studies using the nanostraw platform may involve improving time resolution and delivery speeds or more quantitative assessment of delivery levels, as the precise flux of molecules in the delivery channel into the cell relative to the channel concentration is currently unknown. This proof-of-concept study used several cell densities and bright indicators to demonstrate a range of delivery conditions, but quantification will require the use of ratiometric dyes. The current time resolution of nanostraw delivery matches Ca^{2+} signaling dynamics in motility and transcription and as the time resolution capabilities of nanostraw delivery increase, faster signaling phenomena in neurons and the immune system can be brought into focus.[34] While Ca^{2+} is notable for the quality of its reporters,[36,37,40] other important signaling ions such as

Zn^{2+} are also candidates for study[42] as they are generally less promiscuous than Ca^{2+}. Beyond ionic signaling, time-resolved signaling spans many orders of magnitude.[43] Nanostraws are uniquely poised to study this entire range of phenomena, as long as the messengers are soluble and mobile in solution. In conclusion, nanostraws are a powerful, generic method to explore questions in second messenger signaling and future applications of time-resolved intracellular delivery.

Author information

Author contributions

A. M. X., S. A. K., and N. A. M. designed experiments. A. M. X., D. S. W., and A. A. performed experiments. A. M. X., S. A. K., and N. A. M. wrote the manuscript.

Funding sources

This project was supported by a Bio-X Interdisciplinary Initiatives Project (IIP), A. M. X. was supported by NSF and NDSEG fellowships. S. A. K. was supported by a fellowship from the Phelan-McDermid Syndrome Foundation.

Acknowledgements

We would like to thank the David Goldhaber-Gordon and Yi Cui labs for use of equipment and the Sarah Heilshorn lab for use of microscopes. Additional experiments were performed at the Stanford Nanofabrication Facility and the Stanford Nano Shared Facilities.

References

1 L. S. Collier, C. M. Carlson, S. Ravimohan, A. J. Dupuy and D. A. Largaespada, *Nature*, 2005, **436**, 272–276.
2 P. H. Huang, E. R. Miraldi, A. M. Xu, V. A. Kundukulam, A. M. Del Rosario, R. A. Flynn, W. K. Cavenee, F. B. Furnari and F. M. White, *Mol. BioSyst.*, 2010, **6**, 1227–1237.
3 K. S. Putt, G. W. Chen, J. M. Pearson, J. S. Sandhorst, M. S. Hoagland, J.-T. Kwon, S.-K. Hwang, H. Jin, M. I. Churchwell, M.-H. Cho, D. R. Doerge, W. G. Helferich and P. J. Hergenrother, *Nat. Chem. Biol.*, 2006, **2**, 543–550.
4 D. M. Dykxhoorn, C. D. Novina and P. A. Sharp, *Nat. Rev. Mol. Cell Biol.*, 2003, **4**, 457–467.
5 K. A. Whitehead, R. Langer and D. G. Anderson, *Nat. Rev. Drug Discovery*, 2009, **8**, 129–138.
6 L. Cong, F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini and F. Zhang, *Science*, 2013, **339**, 819–823.
7 M. A. Lemmon and J. Schlessinger, *Cell*, 2010, **141**, 1117–1134.
8 B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter, *Molecular Biology of the Cell*, 4th edn, 2002.
9 D. J. Stephens and R. Pepperkok, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 4295–4298.
10 I. Y. Wong, B. D. Almquist and N. A. Melosh, *Mater. Today*, 2010, **13**, 14–22.
11 T. Kim and J. Eberwine, *Anal. Bioanal. Chem.*, 2010, **397**, 3173–3178.
12 F. D. Urnov, J. C. Miller, Y.-L. Lee, C. M. Beausejour, J. M. Rock, S. Augustus, A. C. Jamieson, M. H. Porteus, P. D. Gregory and M. C. Holmes, *Nature*, 2005, **435**, 646–651.
13 E. S. Boyden, F. Zhang, E. Bamberg, G. Nagel and K. Deisseroth, *Nat. Neurosci.*, 2005, **8**, 1263–1268.
14 F. G. Kuruvilla, A. F. Shamji, S. M. Sternson, P. J. Hergenrother and S. L. Schreiber, *Nature*, 2002, **416**, 653–657.
15 J. Lehar, B. R. Stockwell, G. Giaever and C. Nislow, *Nat. Chem. Biol.*, 2008, **4**, 674–681.
16 B. R. Stockwell, *Nature*, 2004, **432**, 846–854.
17 D. Warther, S. Gug, A. Specht, F. Bolze, J. F. Nicoud, A. Mourot and M. Goeldner, *Bioorg. Med. Chem.*, 2010, **18**, 7753–7758.
18 R. S. Zucker, *Cell Calcium*, 1993, **14**, 87–100.
19 B. C. Pressman, *Annu. Rev. Biochem.*, 1976, **45**, 501–530.
20 W. Kim, J. K. Ng, M. E. Kunitake, B. R. Conklin and P. Yang, *J. Am. Chem. Soc.*, 2007, **129**, 7228–7229.
21 T. E. McKnight, A. V. Melechko, G. D. Griffin, M. A. Guillorn, V. I. Merkulov, F. Serna, D. K. Hensley, M. J. Doktycz, D. H. Lowndes and M. L. Simpson, *Nanotechnology*, 2003, **14**, 551.
22 A. K. Shalek, J. T. Robinson, E. S. Karp, J. S. Lee, D. R. Ahn, M. H. Yoon, A. Sutton, M. Jorgolli, R. S. Gertner, T. S. Gujral, G. MacBeath, E. G. Yang and H. Park, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 1870–1875.
23 J. J. VanDersarl, A. M. Xu and N. A. Melosh, *Nano Lett.*, 2012, **8**, 3881–3886.
24 R. E. Dolmetsch, K. Xu and R. S. Lewis, *Nature*, 1998, **392**, 933–936.
25 P. De Koninck and H. Schulman, *Science*, 1998, **279**, 227–230.
26 J. J. VanDersarl, A. M. Xu and N. A. Melosh, *Lab Chip*, 2011, **11**, 3057–3063.
27 N. P. Christelle, *J. Phys.: Condens. Matter*, 2015, **27**, 233103.
28 H. Persson, Z. Li, J. O. Tegenfeldt, S. Oredsson and C. N. Prinz, *Sci. Rep.*, 2015, **5**, 18535.
29 S. Bonde, N. Buch-Månson, K. R. Rostgaard, T. K. Andersen, T. Berthing and K. L. Martinez, *Nanotechnology*, 2014, **25**, 362001.
30 A. M. Xu, A. Aalipour, S. Leal-Ortiz, A. H. Mekhdjian, X. Xie, A. R. Dunn, C. C. Garner and N. A. Melosh, *Nat. Commun.*, 2014, **5**(5), 3613.
31 A. Aalipour, A. M. Xu, S. Leal-Ortiz, C. C. Garner and N. A. Melosh, *Langmuir*, 2014, **30**, 12362–12367.
32 X. Xie, A. M. Xu, S. Leal-Ortiz, Y. Cao, C. C. Garner and N. A. Melosh, *ACS Nano*, 2013, **7**, 4351–4358.
33 P. F. Baker, D. E. Knight and J. A. Umbach, *Cell Calcium*, 1985, **6**, 5–14.
34 D. E. Clapham, *Cell*, 2007, **131**, 1047–1058.
35 N. L. Allbritton, T. Meyer and L. Stryer, *Science*, 1992, **258**, 1812–1815.
36 A. Miyawaki, O. Griesbeck, R. Heim and R. Y. Tsien, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 2135–2140.

37 A. Miyawaki, J. Llopis, R. Heim, J. M. McCaffery, J. A. Adams, M. Ikura and R. Y. Tsien, *Nature*, 1997, **388**, 882–887.
38 T. Geng and C. Lu, *Lab Chip*, 2013, **13**, 3803–3821.
39 A. H. Faraji and P. Wipf, *Bioorg. Med. Chem.*, 2009, **17**, 2950–2962.
40 T.-W. Chen, T. J. Wardill, Y. Sun, S. R. Pulver, S. L. Renninger, A. Baohan, E. R. Schreiter, R. A. Kerr, M. B. Orger and V. Jayaraman, *Nature*, 2013, **499**, 295–300.
41 K. R. Gee, K. Brown, W. U. Chen, J. Bishop-Stewart, D. Gray and I. Johnson, *Cell Calcium*, 2000, **27**, 97–106.
42 B. Chaigne-Delalande and M. J. Lenardo, *Trends Immunol.*, 2014, **35**, 332–344.
43 B. Novák and J. J. Tyson, *Nat. Rev. Mol. Cell Biol.*, 2008, **9**, 981–991.