Droplet-based microfluidic analysis and screening of single plant cells

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Abstract

Droplet-based microfluidics has been used to facilitate high-throughput analysis of individual prokaryote and mammalian cells. However, there is a scarcity of similar workflows applicable to rapid phenotyping of plant systems where phenotyping analyses typically are time-consuming and low-throughput. We report on-chip encapsulation and analysis of protoplasts isolated from the emergent plant model Marchantia polymorpha at processing rates of >100,000 cells per hour. We use our microfluidic system to quantify the stochastic properties of a heat-inducible promoter across a population of transgenic protoplasts to demonstrate its potential for assessing gene expression activity in response to environmental conditions. We further demonstrate on-chip sorting of droplets containing YFP-expressing protoplasts from wild type cells using dielectrophoresis force. This work opens the door to droplet-based microfluidic analysis of plant cells for applications ranging from high-throughput characterisation of DNA parts to single-cell genomics to selection of rare plant phenotypes.

Introduction

In light of recent advances in DNA synthesis and construct assembly, phenotyping of genetic circuits is on track to becoming limiting to the rate of scientific progress. This is particularly true for plant sciences, where the time required for generation of transgenic organisms ranges from months to years. Protoplasts; individual cells whose wall has been removed through mechanical or enzymatic means, offer an alternative to analysis of plant tissues and open up the possibility of high-throughput phenotyping of single cells [1]. Introduction of DNA into protoplasts by electroporation [2-7], PEG-based transfection [8, 9], or particle bombardment [10] has proven a valuable approach to transient and stable transformation of nuclear and organellar genomes, in particular for plants not amenable to Agrobacterium-mediated transgene delivery. Protoplasts have furthermore been used to overcome barriers...
of sexual incompatibility in generating hybrid plants with novel properties [11]. Following transformation or somatic hybridization, whole plants can be regenerated from individual protoplasts through tissue culture [12]. In addition, protoplasts have become recognized as convenient experimental systems for studying aspects of plant cell ultrastructure, genetics, and physiology [13]. However, to date protoplasts have been extracted and analysed in bulk, limiting their use.

Recently, droplet-based microfluidics has gained increasing popularity as a platform for high-throughput culture, manipulation, sorting, and analysis of up to millions of individual cells under diverse conditions [14–18]. This approach is based on pico- to nanolitre-volume aqueous microdroplets which spatially separate individual cells from one another during processing. To date, droplet-based microfluidics has primarily been applied to bacteria [19–22], unicellular eukaryotes [22–24], and non-adhesive mammalian cells [25, 26]. The prospect of utilizing this platform for characterization and screening of individual plant protoplasts is highly attractive: high-throughput screening of whole plants is substantially limited by their slow growth and size. By contrast, millions of plant protoplasts may be processed in a matter of hours using droplet-based microfluidics, which may prepare regeneration of only pre-selected protoplasts into whole plants.

Microfluidic devices have been applied for the collection and lysis [27], culture [28], chemically-induced fusion [29], electrofusion [30], regeneration [31], and developmental characterization [32] of plant protoplasts. However, platforms for the high-throughput characterization or sorting of individual plant protoplast based on their level of gene expression have been limited to date. One group has explored this approach and used optical tweezers to displace non-encapsulated plant protoplasts in a microfluidic chip, but has not demonstrated successful sorting [33]. While fluorescence-activated cell sorting (FACS) has been applied to sorting of plant cells [34–36], FACS is relatively expensive and not available for many laboratories. Moreover, debris generated during enzymatic treatment of plant tissue has been found to clog the instrument [35]. Taken together with the fragility of plant protoplasts [36], instrument clogging markedly compromises sample injection speed, lowering the rate of events analysed per second. In addition to alleviating these issues, droplet based microfluidics allows the compartmentalization of single cells, thus opening the possibility of rapid prototyping of novel biochemical pathways [22, 37–39].

In this paper, we demonstrate high-throughput characterization and sorting of plant protoplasts encapsulated individually in aqueous microdroplets, based on the genetic expression of a fluorescent reporter protein. We use protoplasts derived from the model plant *Marchantia polymorpha* [40], which combines a simple genomic structure [41, 42] with ease of handling [43] and robustness of regeneration in absence of supplemented plant hormones [44]. We enzymatically isolate *M. polymorpha* protoplasts from adult thalli, and encapsulate them via a flow-focusing microfluidic device. An optical detection setup integrated into the microfluidic channel allows high-throughput quantification of chlorophyll autofluorescence or promoter-controlled YFP fluorescence emitted by individual encapsulated protoplasts. We demonstrate how this droplet-based microfluidic system can be used to rapidly measure the stochastic properties of an inducible plant promoter over a population of individual plant protoplasts. We furthermore show this system is capable of automated sorting of individual encapsulated protoplasts based on their YFP fluorescence intensity. Facilitating high-throughput screening and enrichment of plant protoplasts based on expression of a fluorescent reporter gene, our microfluidic system streamlines the identification and isolation of desired genetic events in plant biology research and modern biotechnology.
Materials and methods

Chemicals, buffers, and media

Unless noted otherwise, chemicals used were obtained from Sigma Aldrich (Haverhill, UK) or Fischer Scientific (Loughborough, UK). DNA primers and Driselase from Basidiomycetes sp. (D8037) were obtained from Sigma Aldrich (Haverhill, UK). Standard molecular biology buffers and media were prepared as described in by Sambrook and Russell [45].

Microfluidic device fabrication

The microfluidic device was fabricated via soft lithography by pouring poly(dimethylsiloxane) (PDMS) along with crosslinker (Sylgard 184 elastomer kit, Dow Corning, Midland, MI, USA; pre-polymer: crosslinker = 10: 1) onto a silicon wafer patterned with SU-8 photoresist [46, 47]. The mixture was degassed in a vacuum dessicator and baked at 75 °C overnight. The devices were peeled from the moulds and holes punched for inlets and outlets using a 1 mm diameter biopsy punch. The channel surface of PDMS was activated using oxygen plasma and attached to a glass slide. To ensure permanent bonding, the complete device was baked overnight at 110 °C. The inner surface of the microchannels was rendered hydrophobic by flowing trifluorooctylethoxysilane through the channels, and the device was baked at 110 °C for 2 h. Electrodes were incorporated into microfluidic chips by inserting a low-melting point indium alloy wire into a punched hole, and melting over a hot plate. Electrical wires were stripped at the end and inserted into the molten indium alloy (see also dx.doi.org/10.17504/protocols.io.ftybnpw).

Binary vector construction

Binary vectors pCRB mpt0 (see Genbank accession No. MF939095) and pCRB PMpHSP17.8 (see Genbank accession No. MF939096) were based on pGreenII [48], and constructed by means of isothermal assembly [49]. To confer hygromycin resistance to transgenic *M. polymorpha*, both binary vectors contained a hygromycin phosphotransferase gene [50] expressed under control of the strong constitutive *MpEF1α* promoter [51]. pCRB further contained an mVenus yellow fluorescent reporter gene [52] under control of PMpEF1α. pCRB PMpHSP17.8 contained an mVenus gene under control of the heat-inducible *MpHSP17.8* promoter [53].

Transformation of *A. tumefaciens*

50 μL aliquots of electrocompetent *A. tumefaciens* GV3101(pMP90) cells containing the pSoup helper plasmid were thawed on ice, mixed with 50–100 ng of DNA at the bottom of a pre-chilled 2 mm gap electroporation cuvette (VWR, Radnor, PA, USA), and kept on ice for 15 min. Electroporation was carried out using an E. coli Pulser Transformation Apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions at 2.50 kV, 5 ms pulse length, and 400 Ω default resistance. 1 mL of liquid SOC medium pre-warmed to 28 °C was then immediately added to each cuvette, and the cells transferred to 15 mL Falcon tubes for recovery over 2–3 h at 28 °C under shaking (ca. 120 rpm). 250 μL of cells were then spread onto LB 1.2%(w/v) agar plates containing 25 μg/mL gentamicin, 5 μg/mL tetracycline, 50 μg/mL rifampicin, and 50 μg/mL kanamycin. Colonies became visible on the agar plates after approximately 2 days of incubation at 28m°C.
Plant materials and growth conditions

*M. polymorpha* Cam-strain plants were grown on B5 medium supplemented with 1.6 g/L vitamins (1/2 B5Vit; G0210, Melford, Ipswich, UK) containing 1.2%(w/v) agar, under continuous white light.

Surface sterilization and germination of *M. polymorpha* spores. *M. polymorpha* sporangia (2 per nuclear transformation to be attempted) were crushed with a polypropylene cell spreader until only small fragments (< 5 mm in diameter) remained visible. Sterile dH$_2$O (1 mL per nuclear transformation) was added, and the tube vortexed vigorously for 30 sec. The crushing and vortexing steps were repeated, the suspension passed through a Falcon 40 μm cell strainer (Corning, Wiesbaden, Germany) to remove plant debris, and 500 μL aliquots of the filtrate transferred into 1.5 mL Eppendorf tubes. Spores were spun down at 13,000 rpm for 1 min, and the supernatant removed without disturbing the pellet. Each pellet was then resuspended into 1 mL of a sterilizing solution prepared by dissolving 1 Milton Mini Sterilizing Tablet (Procter & Gamble, Cincinnati, OH, USA) in 25 mL of sterile dH$_2$O. The tubes were shaken at room temperature for 20 min at 200 rpm. Surface-sterilized spores were then pelleted by centrifugation as above and washed by 1 mL of sterile dH$_2$O. The spore content of each tube was resuspended in 100 μL of sterile dH$_2$O and spread on two 1/2 B5Vit 1.2%(w/v) agar plates. The plates were sealed and kept inverted under white fluorescent light at 23 °C as described above. Small thalli were visible under a stereomicroscope after approximately 1 week.

Nuclear transformation of *M. polymorpha* sporelings

2–3 colonies of *A. tumefaciens* GV3101 (pMP90,pSoup) carrying a binary plasmid of interest were used to inoculate 4 mL of selective LB medium supplemented by 100 μM acetosyringone, and the culture incubated overnight at 28 °C under shaking (ca. 120 rpm). 1 mL of the overnight culture was used to inoculate 4 mL of selective 1/2 B5Vit medium supplemented by 100 μM acetosyringone, 0.1%(w/v) casamino acids, 0.03%(w/v) glutamine, and 2%(w/v) sucrose (1/2 B5VitAcSuc). The diluted culture was incubated at 28 °C for 4 h under shaking (ca. 120 rpm). Germinating spores of *M. polymorpha* on day 6 after surface sterilization were harvested by adding 2 mL of 1/2 B5VitSuc (equals 1/2 B5VitAcSuc without acetosyringone) to each plate, resuspending germinating spores in the liquid, through scraping them off the agar using a polypropylene cell spreader, and transferring the suspension to a 50 mL Falcon tube using a pipette. For each transformation, a suspension of germinating spores corresponding to the content of 2 agar plates (i.e. 2 sporangia) was diluted into 50 mL of 1/2 B5VitAcSuc in a baffled 250 mL Erlenmeyer shaking flask. Following addition of 1 mL of transgenic *A. tumefaciens* GV3101 (pMP90,pSoup), subcultured in 1/2 B5VitAcSuc as described above, each flask was shaken at 150 rpm for 48 h under white fluorescent light at 23 °C. After co-cultivation, spores were rescued by passing the suspension through a Falcon 40 μm cell strainer (Corning, Wiesbaden, Germany). Collected spores were washed by 200 mL of 100 μg/mL cefotaxime in sterile dH$_2$O to remove *A. tumefaciens*, and spread on 1/2 B5VitAcSuc 1.2%(w/v) agar plates containing 100 μg/mL cefotaxime and 20 μg/mL hygromycin. The spore content of a single shaking flask was distributed to 3–4 agar plates after collection and washing. Transgenic thalli were observed after 1–2 weeks under white fluorescent light at 2 3˚C.

Protoplast preparation

Protoplasts were isolated from *M. polymorpha* thalli as previously described [40], with modifications: thalli were vacuum-infiltrated by 1/2 B5 containing 2%(w/v) Driselase and 6%(w/v) Mannitol for 10 min in a glass beaker, and subsequently incubated in the dark at room
temperature for 5 h. The beaker was then gently swirled for 30 sec to aid protoplast release, and the protoplast-containing suspension passed through a Falcon 40 μm cell strainer to remove debris. Protoplasts were isolated from A. thaliana as previously described [54].

**Protoplast encapsulation in microfluidic droplets**

Protoplasts in the aqueous phase were encapsulated into droplets using a flow-focusing microfluidic device: the protoplast suspension was loaded into a 500 μL Hamilton Gas-tight syringe (Hamilton Robotics, Reno, NV, USA). The fluorinated oil used as the continuous phase (3M Novec 7500 Engineered Fluid with 2.5% PicoSurf 1 surfactant, Sphere Fluidics, Cambridge, UK) was loaded in another syringe and both syringes were connected to the respective inlets of the flow-focusing device (nozzle dimensions: 40 μm x 40 μm x 50 μm) with fine bore polyethylene tubing (ID = 0.38 mm, OD = 1.09 mm, Smiths Medical International, Luton, UK). Using syringe pumps (PHD 2000 Infusion, Harvard Apparatus, Holliston, MA), the two solutions were injected simultaneously in the device. The oil phase was injected at a rate of 500 μL/h and the aqueous phase at a rate of 300 μL/h. The generated droplets were collected, through tubing connected to the outlet, into a syringe.

**Bright-field and fluorescence microscopy**

Microdroplet formation was monitored using a Phantom V72 fast camera (Vision Research, Wayne, NJ, USA) mounted on an inverted microscope (IX71, Olympus, Tokyo, Japan). Videos of the encapsulation procedure were captured using the supplied Phantom software. Protoplasts encapsulated in microdroplets were imaged using an inverted microscope (IX71, Olympus, Tokyo, Japan). Chlorophyll fluorescence was excited at 642–682 nm and collected at 603.5–678.5 nm. YFP fluorescence was excited at 488–512 nm and collected at 528.5–555.5 nm.

**On-chip fluorescence measurements and sorting of encapsulated protoplasts**

To measure protoplasts fluorescence in each microdroplet, a fixed 491 nm wavelength laser (Cobolt AB, Solna, Sweden) was shaped into a light sheet at 50 mV. The laser was focused through an UPlanFL N 20x microscope objective and directed to the microfluidic chip placed on the stage of an inverted microscope (IX71, Olympus, Tokyo, Japan). Fluorescence detection was carried out by a custom multi-part optical instrument. All filters used in this setup were purchased from Semrock (Rochester, NY, USA). Notably, emitted fluorescence was filtered through a 495 nm long-pass filter to eliminate the 491 nm excitation band. Fluorescence was recorded by a PMT (H8249, Hamamatsu Photonics, Shizuoka, Japan), and the data collected was sent to a computer through a DAQ data acquisition card (National Instruments, Austin, TX, USA). The program LabVIEW (National Instruments, Austin, TX, USA) was used to monitor and analyse the data. A microfluidic device was used for sorting YFP-expressing protoplasts in microdroplets: as the microdroplets passed through the objective field of view, they were illuminated by a 491 nm laser. Emitted fluorescence filtered through a 528.5–555.5 nm YFP band-pass filter was collected by the PMT and triggered a pulse generator connected to a high-voltage power supply. The resulting electrode pulse (200 V) deformed YFP-positive microdroplets and targeted them to a small ‘positive’ channel for collection. If the microdroplet was empty or contained protoplast lacking detectable YFP, the PMT sent no signal and the microdroplet passed through the larger ‘negative’ channel.
Data

Extended protocols can be found at dx.doi.org/10.17504/protocols.io.ftnbnme and dx.doi.org/10.17504/protocols.io.ftybnpw.

Results and discussion

Isolated *M. polymorpha* protoplasts were encapsulated in microdroplets on a flow-focusing microfluidic device based on [18] (Fig 1A). The aqueous protoplasts suspension flowed perpendicularly to two streams of fluorinated carrier oil containing PicoSurf1 non-ionic surfactant. The two phases intersected at the ‘flow-focusing junction’, as the oil streams enveloped the droplet that budded off from the aqueous stream (Fig 1B). The density of *M. polymorpha* protoplasts was adjusted to ensure microdroplets contained no more than one protoplast each (Fig 1C), which is important for accurate quantification of cellular fluorescence intensity. The
same approach was also successful for encapsulation of the widely used angiosperm model *Arabidopsis thaliana* (S1 Fig).

While encapsulated, protoplasts remained intact over a period of at least 12 hours (Fig 2). To quantify chlorophyll autofluorescence in individual encapsulated protoplasts, an optical setup was integrated to the system (Fig 3). Each microdroplet was re-injected into a microfluidic flow channel continuously exposed to a 491 nm laser beam. Fluorescence emitted from excited protoplasts passed through a 633 nm longpass filter and the signal was collected by a photomultiplier tube (PMT; S1 Video). Using this experimental approach, the fluorescence of each protoplast was quantified for up to 115,200 individual protoplasts per hour. This observation suggests that high-throughput quantification of chlorophyll fluorescence using our microfluidic setup can be utilized for assessment of the quality of a protoplast preparation. The same experimental approach was also used for quantification of reporter protein fluorescence in individual plant cells, as illustrated by protoplasts derived from transgenic mpt0

**Fig 2. Protoplast stability after encapsulation.** (A) Bright field and (B) chlorophyll fluorescence micrographs of individual *M. polymorpha* protoplast encapsulated in microdroplets.

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M. polymorpha constitutively expressing mVenus [41] yellow fluorescent protein (YFP) under control of the strong constitutive MpEF1α promoter [42] (Fig 4).

As the next step, our system was applied for the analysis of the stochastic activity of an inducible promoter across a population of individual plant cells. For this purpose, transgenic PMpHSP17.8 lines of M. polymorpha were generated, which expressed mVenus yellow fluorescent protein (YFP) [52] under control the endogenous heat-responsive MpHSP17.8 promoter. It was previously shown that incubation of transgenic M. polymorpha at 37˚C for 1 h induced a PMpHSP17.8-controlled targeted gene by approximately 700-fold [53].

To measure the stochastic properties of this promoter, transgenic PMpHSP17.8 M. polymorpha thalli were either subjected to (i) 2 h at 37˚C followed by 2 h at room temperature or to (ii) 4 h at room temperature (control). Protoplasts isolated from heat-shocked plants exhibited significantly higher levels of YFP activity compared to the Control (p < 2.2e-16, 95% CI [-0.2, -0.13]). This result illustrates the power of our microfluidic system to quantify stochastic properties of plant promoters as a function of environmental conditions.
An even more powerful application of our microfluidic platform is sorting of individual encapsulated protoplasts based on their level of expression of a target reporter gene. This allows single plant cells to be pre-screened for downstream sequencing and/or regeneration of whole plants. For this purpose, a microdroplet-based microfluidic sorting system was developed (Fig 6A): two oil flow-focusing channels allowed the spacing between microdroplet to be controlled by flow-rate adjustment. Microdroplet sorting was implemented by a pair of electrodes generating a dielectrophoretic force applied to the microdroplet. When the electrodes were off, the microdroplets were pushed into the “negative” channel due to its lower fluidic resistance compared to the “positive” channel. Switching the electrodes on steered the individual microdroplets into the “positive” channel through dielectrophoretic force. The generation of an electrode pulse was dependent on the fluorescence intensity emitted from each microdroplet: microdroplets were steered to the "positive" channel only if they contained a

Fig 4. Reporter fluorescence quantification on encapsulated protoplasts. (A) Bright field and (B) mVenus fluorescence micrograph of an individual encapsulated protoplast derived from transgenic mpt0 M. polymorpha constitutively expressing mVenus. (C) Representative PMT readout of mVenus fluorescence intensity recorded on chip over 120 s. Each line represents an individual encapsulated protoplast.

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protoplast expressing YFP above-threshold levels of 1.3 arbitrary fluorescence units (AFU; S2 Video). The platform was tested for using microdroplets containing protoplasts isolated from either wild type or transgenic mpt0 M. polymorpha. Protoplast from both populations were pooled together and reinjected into the sorting device (Fig 6B). Sorting successfully separated mVenus-expressing mpt0 protoplasts from ruptured cells, wild type protoplasts and empty droplets. Fig 6C shows representative microdroplets collected through the “positive” and “negative” channels. All the microdroplets collected through the “positive” channel contained mVenus-expressing mpt0 protoplasts. Among the “negative” channel we observed wild type protoplasts and a considerable number of empty droplets (Fig 6C and S2 Fig). This result showed our microfluidic platform capable of high-throughput selection of desired events across large populations of genetically diverse individual plant cells.

While microdroplet-based microfluidics has previously been applied to unicellular photosynthetic eukaryotes [21] the work presented here allows for rapid screening of synthetic circuits designed for operation in multicellular plants. Although further work will be required to assess the capacity for regeneration of whole plants from individual sorted protoplasts, this work provides a fundament for the development of techniques reducing the time and cost involved in the screening of transgenic plants, by alleviating the need to maintain and cultivate large quantities of callus prior to selection.

**Conclusions**

We have developed a droplet-based microfluidic platform for high-throughput characterization of plant protoplasts. Our device is capable of quantifying chlorophyll and GFP fluorescence of
individual encapsulated cells as a function of genetic circuit activity or in response to environmental stimuli. This workflow allows collection of substantial amounts of biological information from comparatively little plant material. We expect our droplet-based microfluidic platform to be applied for screening of synthetic genetic circuits as well as of mutagenized and enhancer trap lines of a variety of plant species. In the future, we envision a microfluidic workflow composed of on-chip transformation, characterization, and fluorescence-based selection of individual plant cells in preparation of targeted regeneration into whole plants. Combined with libraries of guide RNAs and gene editing tools such as CRISPR-Cas9 nuclease, this workflow promises to greatly accelerate academic and industrial research in modern plant biotechnology.

Fig 6. Sorting of *M. polymorpha* protoplasts. (A) Schematic representation of a platform for microfluidic sorting of encapsulated protoplasts. (B) Bright field and fluorescence micrographs of adjacent microdroplets containing protoplasts derived from wild-type and transgenic mp0 M. polymorpha, respectively. (C) Bright field and fluorescence micrographs of microdroplets sorted into positive and negative channels based on their mVenus fluorescence intensity.

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Supporting information

S1 Fig. Encapsulation of A. thaliana protoplasts. A) Bright field and (B) chlorophyll fluorescence micrographs of individual A. thaliana leaf protoplasts encapsulated in microdroplets. (C) Representative photomultiplier tube (PMT) readout of chlorophyll fluorescence intensity represented as arbitrary fluorescent units (AFU) recorded over 17.5 s. Each line represents an individual encapsulated protoplast.

(TIF)

S2 Fig. Sorting of M. polymorpha protoplasts. Bright field and fluorescence micrographs of microdroplets sorted into positive and negative channels based on their mVenus fluorescence intensity. Scale bars; 50 μm.

(PDF)

S1 Video. Encapsulation of Arabidopsis thaliana protoplasts.

(ZIP)

S2 Video. Sorting of M. polymorpha protoplasts expressing YFP.

(ZIP)

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References

1. Sheen J. Signal transduction in maize and Arabidopsis mesophyll protoplasts. Plant Physiol. 2001; 127: 1466–1475. PMID: 11743090

2. Fromm M, Taylor LP, Walbot V. Expression of Genes Transferred into Monocot and Dicot Plant-Cells by Electroporation. Proc Natl Acad Sci U S A. 1985; 82: 5824–5828. https://doi.org/10.1073/Pnas.82.17.5824 PMID: 3862099

3. Ou-Lee T-M, Turgon R, Wu R. Expression of a foreign gene linked to either a plant-virus or a Drosophila promoter, after electroporation of protoplasts of rice, wheat, and sorghum. Proc Natl Acad Sci. 1986; 83: 6815–6819. https://doi.org/10.1073/pnas.83.18.6815 PMID: 16893757

4. Hauptmann RM, Ozias-Akins P, Vasil V, Tabaeizadeh Z, Rogers SG, Horsch RB, et al. Transient expression of electroporated DNA in monocotyledinous and dicotyledinous species. Plant Cell Rep. 1987; 6: 265–270. https://doi.org/10.1007/BF00271995 PMID: 24248756

5. Negrutiu I, Shillito R, Potrykus I, Biasini G, Sala F. Hybrid genes in the analysis of transformation conditions. Plant Mol Biol. 1987; 8: 363–373. https://doi.org/10.1007/BF00015814 PMID: 24301258
6. Nishiguchi M, Sato T, Motoyoshi F. An improved method for electroporation in plant protoplasts: infection of tobacco protoplasts by tobacco mosaic virus particles. Plant Cell Rep. 1987; 6: 90–93. PMID: 2248484
7. Jones H, Ooms G, Jones MGK. Transient gene expression in electroporated Solanum protoplasts. Plant Mol Biol. 1989; 13: 503–511. https://doi.org/10.1007/BF00027310 PMID: 2491668
8. Krens FA, Molendijk L, Wullems GJ, Schilperoort RA. In vitro transformation of plant protoplasts with Ti-plasmid DNA. Nature. Cambridge: Cambridge University Press; 1982; 296: 72–74. https://doi.org/10.1038/296072a0
9. Potrykus I, Paszkowski J, Saul MW, Petruska J, Shillito RD. Molecular and general genetics of a hybrid foreign gene introduced into tobacco by direct gene transfer. MGG Mol Gen Genet. 1985; 199: 169–177. https://doi.org/10.1007/BF00330255 PMID: 3860712
10. Huang FC, Klaus S, Herz S, Zou Z, Koop HU, Golds T. Efficient plastid transformation in tobacco using the aphA-6 gene and kanamycin selection. Mol Genet Genomics. 2002; 268: 19–27. https://doi.org/10.1007/s00438-002-0738-6 PMID: 12242495
11. Waara S, Glimelius K. The potential of somatic hybridization in crop breeding. Euphytica. 1995; 85: 217–233. https://doi.org/10.1007/BF0023951
12. Roest S, Gilissen W. Plant Regeneration from Protoplasts: A Literature Review. Acta Bot Neerl. 1989; 38: 1–23.
13. Davey MR, Anthony P, Power JB, Lowe KC. Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv. 2005; 23: 131–171. https://doi.org/10.1016/j.biotechadv.2004.09.008 PMID: 15694124
14. Autebert J, Coudert B, Bidard FC, Pierga JY, Descroix S, Malaquin L, et al. Microfluidic: An innovative tool for efficient cell sorting. Methods. Elsevier Inc.; 2012; 57: 297–307. https://doi.org/10.1016/j.ymeth.2012.07.002 PMID: 22796377
15. Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD, Heyman JA. Single-cell analysis and sorting using droplet-based microfluidics. Nat Protoc. 2013; 8: 870–891. https://doi.org/10.1038/nprot.2013.046 PMID: 23558786
16. Wang BL, Ghaderi A, Zhou H, Agresti J, Weitz DA, Fink GR, et al. Microfluidic high-throughput culturing of single cells for selection based on extracellular metabolite production or consumption. Nat Biotechnol. Nature Publishing Group; 2014; 32: 473–478. https://doi.org/10.1038/nbt.2857 PMID: 24705516
17. Wyatt Shields C IV, Reyes CD, López GP. Microfluidic cell sorting: a review of the advances in the separation of cells from debulking to rare cell isolation. Lab Chip. Royal Society of Chemistry; 2015; 15: 1230–1249. https://doi.org/10.1039/C4LC01246A PMID: 25598308
18. Best RJ, Lyczkowski JJ, Abalde-Cela S, Yu Z, Abell C, Smith AG. Label-Free Analysis and Sorting of Microalgae and Cyanobacteria in Microdroplets by Intrinsic Chlorophyll Fluorescence for the Identification of Fast Growing Strains. Anal Chem. American Chemical Society; 2016; 88: 10445–10451. https://doi.org/10.1021/acs.analchem.6b02364 PMID: 27677315
19. Martin K, Henkel T, Baier V, Grodrian A, Schön T, Roth M, et al. Generation of larger numbers of separated microbial populations by cultivation in segmented-flow microdevices. Lab Chip. 2003; 3: 202–207. https://doi.org/10.1039/b301258c PMID: 15100775
20. Huebner A, Srissa-Art M, Holt D, Abell C, Hoffelder F, DeMello AJ, et al. Quantitative detection of protein expression in single cells using droplet microfluidics. Chem Commun (Camb). 2007; 2: 1218–20. https://doi.org/10.1039/b618570c PMID: 17356761
21. Lim JW, Shin KS, Moon J, Lee SK, Kim T. A Microfluidic Platform for High-Throughput Screening of Small Mutant Libraries. Anal Chem. 2016; 88: 5234–42. https://doi.org/10.1021/acs.analchem.6b00317 PMID: 27104360
22. Gach PC, Shih SCC, Sustarich J, Keasling JD, Hillson NJ, Adams PD, et al. A Droplet Microfluidic Platform for Automating Genetic Engineering. ACS Synth Biol. 2016; 5: 426–33. https://doi.org/10.1021/acssynbio.6b00011 PMID: 26830031
23. Oh H-J, Kim S-H, Baek J-Y, Seong G-H, Lee S-H. Hydrodynamic micro-encapsulation of aqueous fluids and cells via “on the fly” photopolymerization. J Micromechanics Microengineering. 2006; 16: 285–291. https://doi.org/10.1088/0960-1317/16/2/013
24. Terekhov SS, Smirnov I V., Stepanova A V., Bobik T V., Mokrushina YA, Ponomarenko NA, et al. Microfluidic droplet platform for ultra-high-throughput single-cell screening of biodiversity. Proc Natl Acad Sci U S A. 2017; 114: 2550–2555. https://doi.org/10.1073/pnas.1621226114 PMID: 28202731
25. Sakai S, Kawabata K, Ono T, Ijima H, Kawakami K. Higher viscous solution induces smaller droplets for cell-enclosing capsules in a co-flowing stream. Biotechnol Prog. 2005; 21: 994–997. https://doi.org/10.1021/bp049600o PMID: 15932285
26. He M, Edgar JS, Jeffries GDM, Lorenz RM, Shelby JP, Chiu DT. Selective encapsulation of single cells and subcellular organelles into picoliter- and femtoliter-volume droplets. Anal Chem. 2005; 77: 1539–1544. https://doi.org/10.1021/ac0480850 PMID: 15762555

27. Hung M-S, Chang J-H. Developing microfluidics for rapid protoplasts collection and lysis from plant leaf. Proc Inst Mech Eng Part N J Nanoeng Nanosyst. 2012; 226: 15–22. https://doi.org/10.1177/174039911444511

28. Ko J-M, Ju J, Lee S, Cha H-C. Tobacco protoplast culture in a polydimethylsiloxane-based microfluidic channel. Protoplasma. 2006; 227: 237–240. https://doi.org/10.1007/s00709-005-0142-2 PMID: 16736262

29. Wu H, Liu W, Tu Q, Song N, Li L, Wang J, et al. Culture and chemical-induced fusion of tobacco mesophyll protoplasts in a microfluidic device. Microfluid Nanofluidics. 2011; 10: 867–876. https://doi.org/10.1007/s10404-010-0720-2

30. Ju J, Ko J-M, Cha H-C, Park JY, Im C-H, Lee S-H. An electrofusion chip with a cell delivery system driven by surface tension. J Micromech Microengineering. 2009; 19: 15004. https://doi.org/10.1088/0960-1317/19/1/015004

31. Bascom CS, Wu S-Z, Nelson K, Oakey J, Bezanilla M. Long-Term Growth of Moss in Microfluidic Devices Enables Subcellular Studies in Development. Plant Physiol. 2016; 172: 28–37. https://doi.org/10.1104/pp.16.00879 PMID: 27406170

32. Zaban B, Liu W, Jiang X, Nick P. Plant Cells Use Auxin Efflux to Explore Geometry. Sci Rep. 2015; 4: 5852. https://doi.org/10.1038/srep05852 PMID: 25068254

33. Landenberger B, Höfemann H, Wadle S, Rohrbach A. Microfluidic sorting of arbitrary cells with dynamic optical tweezers. Lab Chip. Royal Society of Chemistry; 2012; 12: 3177. https://doi.org/10.1039/c2lc21099a PMID: 22767208

34. Redenbaugh K, Ruzin S, Bartholomew J, Bassham JA. Characterization and Separation of Plant Protoplasts Via Flow Cytometry and Cell Sorting. Zeitschrift für Pflanzenphysiologie. Urban & Fischer; 1994; 107: 65–80. https://doi.org/10.1007/S00443-028X(11)80010-3

35. Bargmann BOR, Birnbaum KD. Fluorescence activated cell sorting of plant protoplasts. J Vis Exp. MyJoVE Corporation; 2010; https://doi.org/10.3791/1673 PMID: 20168296

36. Grønlund JT, Eyres A, Kumar S, Buchanan-Wollaston V, Gifford ML. Cell specific analysis of Arabidopsis leaves using fluorescence activated cell sorting. J Vis Exp. MyJoVE Corporation; 2012; https://doi.org/10.3791/4214 PMID: 23070217

37. Gulati S, Rouilly V, Niu X, Chappell J, Kitney RI, Edel JB, et al. Opportunities for microfluidic technologies in synthetic biology. J R Soc Interface. The Royal Society; 2012 Suppl 4: S493–506. https://doi.org/10.1098/rsif.2012.0083.focus PMID: 19474079

38. Vinuselvi P, Park S, Kim M, Park JM, Kim T, Lee SK. Microfluidic technologies for synthetic biology. Int J Mol Sci. Multidisciplinary Digital Publishing Institute (MDPI); 2011; 12: 3576–3593. https://doi.org/10.3390/ijms12063576 PMID: 21747695

39. Abalde-Cela S, Gould A, Liu X, Kazamia E, Smith AG, Abell C. High-throughput detection of ethanol-producing cyanobacteria in a microdroplet platform. J R Soc Interface. 2015; Available: http://rsif.rsgrouppublishing.org/content/12/106/20150216

40. Bowman JL, Kohchi T, Yamato KT. The “sneak” preview of the Marchantia polymorpha genome. BSJ Rev. 2012; 3: 71–83.

41. Bowman JL, Kohchi T, Yamato KT, Jenkins J, Shu S, Ishizaki K, et al. Insights into Land Plant Evolution Gathered from the Marchantia polymorpha Genome. Cell. Elsevier; 2017; 171: 287–304.e15. https://doi.org/10.1016/j.cell.2017.09.030 PMID: 28995561

42. Ishizaki K, Nishihamura R, Yamato KT, Kohchi T. Molecular Genetic Tools and Techniques for Marchantia polymorpha Research. Plant Cell Physiol. 2016; 57: 262–270. https://doi.org/10.1093/pcp/pcv097 PMID: 26116421

43. Bopp M, Vicktor R. Protoplasts of Marchantia polymorpha and its Development. Plant Cell Physiol. 1988; 29: 497–501.

44. Sambrook J, Russell DD. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press; 2001.

45. Qin D, Xia Y, Whitesides GM. Rapid prototyping of complex structures with feature sizes larger than 20 μm. Adv Mater. 1996; 917–919. https://doi.org/10.1002/adma.19960081110
47. Duffy DC, McDonald JC, Schueller OJA, Whitesides GM. Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane). Anal Chem. 1998; 70: 4974–4984. https://doi.org/10.1021/ac980656z PMID: 21644679

48. Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol Biol. 2000; 42: 819–832. PMID: 10890530

49. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison C a, Smith HO, et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009; 6: 343–5. https://doi.org/10.1038/nmeth.1318 PMID: 19363495

50. Ishizaki K, Chiyoda S, Yamato KT, Kohchi T. Agrobacterium-Mediated Transformation of the Haploid Liverwort Marchantia polymorpha L., an Emerging Model for Plant Biology. Plant Cell Physiol. 2008; 49: 1084–1091. https://doi.org/10.1093/pcp/pcn085 PMID: 18535011

51. Althoff F, Kopischke S, Zobell O, Ide K, Ishizaki K, Kohchi T, et al. Comparison of the MpEF1α and CaMV35 promoters for application in Marchantia polymorpha overexpression studies. Transgenic Res. 2014; 23: 235–244. https://doi.org/10.1007/s11248-013-9746-z PMID: 24036909

52. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol. 2002; 20: 87–90. https://doi.org/10.1038/nbt0102-87 PMID: 11753368

53. Nishihama R, Ishida S, Urawa H, Kamei Y, Kohchi T. Conditional Gene Expression/Deletion Systems for Marchantia polymorpha Using its Own Heat-Shock Promoter and Cre/lox P-Mediated Site-Specific Recombination. Plant Cell Physiol. 2016; 57: 271–280. https://doi.org/10.1093/pcp/pcv102 PMID: 26148498

54. Yoo S-D, Cho Y-H, Sheen J. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc. Nature Publishing Group; 2007; 2: 1565–1572. https://doi.org/10.1038/nprot.2007.199 PMID: 17585298