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Review**Microfluidic platforms for plant cells studies**Amir Sanati Nezhad^{1,2}

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*Amir Sanati Nezhad**Submitted to Lab on Chip***Abstract**

Conventional methods of plant cell analysis rely on growing plant cells in soil pots or agarose plates, followed by screening the plant phenotypes in traditional greenhouses and growth chambers. These methods are usually costly, need a large number of experiments, suffer from low spatial resolution and disorderly growth behavior of plant cells, with lack of ability to locally and accurately manipulate the plant cells. Microfluidic platforms take advantage of miniaturization for handling small volume of liquids and providing a closed environment, with the purpose of *in-vitro* single cell analysis and characterizing cell response to external cues. These platforms have shown their ability for high-throughput cellular analysis with increased accuracy of experiments, reduced cost and experimental times, versatility in design, ability for large-scale and combinatorial screening, and integration with other miniaturized sensors. Despite extensive research on animal cells within microfluidic environments for high-throughput sorting, manipulation and phenotyping studies, the application of microfluidics for plant cells studies has not been accomplished yet. Novel devices such as RootChip, RootArray, TipChip, and PlantChip developed for plant cells analysis, with high spatial resolution on a micrometer scale mimicking the internal microenvironment of plant cells, offering preliminary results on the capability of microfluidics to conquer the constraints of conventional methods. These devices have been used to study different aspects of plant cell biology such as gene expression, cell biomechanics, cellular mechanism of growth, cell division, and cells fusion. This review emphasizes the advantages of current microfluidic systems for plant science studies, and discusses future prospects of microfluidic platforms for characterizing plant cells response to diverse external cues.

1 Introduction

Micro-electro-mechanical systems (MEMS) technology has been widely used for biological studies in the last decade, which led to the development of new area called BioMEMS. Today, BioMEMS are extensively used in different applications such as drug development, biosensing, synthesizing *in-vitro* organs, and particularly, cellular studies.¹⁻⁴ Microfluidic platforms, that provide closed microenvironments, take advantage of miniaturization for handling small volume of liquids for *in-vitro* single cell analysis and characterizing cell response to different external cues.⁵⁻⁷ In addition, the ability of high-throughput cellular analysis increases the accuracy of experiments, and reduces the cost and experimental times. Despite extensive research on animal cells within microfluidic environments, including high throughput sorting, manipulation, phenotyping studies, and external stimulation, their application for plant cell studies has not been accomplished yet.⁸⁻¹⁰

Conventional plant cell analysis methods rely on culturing the seeds, growing the plant cells in soil pots or agarose plates, followed by screening the plant phenotypes in traditional greenhouses and growth chambers. These methods are usually costly, need a large number of experiments, and suffer from information loss during the monitoring of plant phenotypic changes due to low temporal resolution. In addition, disorderly growth behavior of plant cells on solid medium makes the detailed analysis of growth impossible. Microfluidics has shown its strength in developing research on plant cell science, since it allows high spatial resolution on a micrometer scale by developing an environment that mimics the internal microenvironment of plant cells. Here, applications and advantages of microfluidic systems for plant science studies is reviewed and future prospects of microfluidics for characterizing plant cells in response to diverse external cues is discussed. Table 1 presents the main microfluidic devices currently developed to study plant cells, as well as the application and advantage of each device to conquer the constraints of conventional methods.

Table 1 Applications and advantages of microfluidic devices used for plant cell studies in comparison with conventional assays

Microfluidic platform	Application	Significance in comparison with conventional assays	References
RootChip	Monitoring free metals concentration and phenotyping of root metabolism for several days	Long term and real-time monitoring of root growth, High-throughput quantitative analysis	Grossmann et al. ¹³
RootArray	Monitoring spatiotemporal gene expression dynamics	Real-time screening of gene expression, Automating the imaging systems reconstructing 3D root shape	Busch et al. ²¹
PlantChip	Quantitative analysis and monitoring phenotypes of immutans mutant for multiple roots	Long term, continuous and concurrent monitoring of roots, shoots, hypocotyl, cotyledon, and even a few leaves	Jiang et al. ²²
TipChip	High throughput chemical or biological simulation of pollen tubes	Providing identical growth condition for a series of individual pollen tubes	Agudelo et al. ²⁴ Sanati Nezhad et al. ²⁵
Arrayed pollen tubes	Long term imaging of pollen tubes with high resolution	pollen tube is confined to a fixed focal plane, allowing accurate microscopic observations	Horade et al. ⁷
ToxChip	Whole cell sensor to detect the presence of pollutants in the environment	Higher accuracy in respect to the previous open assays. Detection of secondary oscillation frequency and its variation under chemical treatment	Sanati Nezhad et al. ³⁸
Local treatment At tip	<i>In vitro</i> Investigating the reorientation of pollen tube growth in response to local chemical gradient	Rapid and reliable change of growth medium at subcellular resolution locally around growing pollen tubes	Yetisen et al. ⁵² Horade et al. ⁵⁷
Local treatment at distal region	understanding the mechanism of root development by differentiating the active and passive transport mechanisms of auxin through observing level of GFP expression	Monitoring the transport mechanisms of auxin in roots in response to local manipulation at subcellular resolution	Meier et al. ⁵⁹
BendingChip	Quantifying the mechanical properties of pollen tube cell wall	Capability of direct bending-based manipulation on pollen tube. Results are independent from the properties of micro-indenter	Sanati Nezhad et al. ⁶⁸
Microgap Chip	Measuring the growth force of pollen tube	Integrating sensors in subcellular size. The sensor prevents redirection of growth, resulting in a conclusive growth force in comparison with off-chip sensors	Sanati Nezhad et al. ²⁷
"Directional Memory" Chip	Investigating the presence of "inner memory" in different plant cells	Capability of systematic testing on plant cells by providing desired subcellular microenvironment	Bibikova et al. ⁷⁶ Held et al. ⁷⁷
Cell division studies	Long term monitoring of plant cells division with minimal damage to cells	Less volumes of media, smaller cell population size, optimal supplying of nutrients, highly spatiotemporal control of microenvironment	Wu et al. ⁷⁹
On-chip electrofusion	Electrofusion of plant protoplasts with the application in gene transfection studies	Superior control on cell manipulation at single cell level	Ju et al. ⁸³

2 Plant-Chips

2.1 RootChip: The microfluidic platform for plant root science

Roots, as important plant compartments for water and nutrients uptake, respond to the change in environmental conditions and are very sensitive to dehydration or physical stimulation. Hence, to provide an *in vitro* platform for studying molecular mechanism of growth, several important parameters need to be taken into account, such as the stability of growth environment, the reproducibility to change environmental condition and multiplexing in order to enhance the efficiency of experimental results and to obtain phenotypic information. Characterizing molecular processes of root elongation requires a microenvironment at cellular resolution with high controllability on altering the root environmental condition. Conventional tools such as perfusion chambers¹¹ and 96-well plates¹² lack the ability for high-throughput and precise environmental changes, respectively. In addition, the off-chip chemical probes assembled to conventional assays are restricted to static analysis of ions with low imaging resolution due to the limited control on localization of the probe. The dynamic analysis of ions using chemical probes needs complex manipulation system. The Rootchip was developed for this purpose, to study the physiology of growing roots in a controllable microenvironment in a high-throughput platform.^{13, 14} *Arabidopsis* seeds germinate in agar-filled conical cylinders made from micropipette tips and elongate gravitropically into the plastic cone for 5 days to reach the access holes, where the root changes its growth orientation from a vertical position to a horizontal state. An embedded bifurcating tree structure flows the growth medium into eight parallel observation chambers toward individual elongating roots with the purpose of real-time monitoring of root growth (Fig 1a). The quantified average growth rate of the roots was comparable with the rate measured in plate assays. In addition, the cytosolic glucose and galactose levels of the roots were monitored in real-time by the fluorescence sensors. The suitability of the platform for long-term experiments was tested by adjusting the channel height within the size range of the root diameter and preventing Z-drift of the root during elongation. The growth length and fluorescence signals of *Arabidopsis* seedlings were successfully monitored over 24 h. The RootChip platform can be used for other plant species after slight adaptation in the design, in order to study the root growth and high-throughput quantitative phenotyping of root metabolism for several days. The capability of temporal

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root perfusion and noninvasive detection of metabolite levels make this system unique for studying uptake at different root zones such as root hair cells or even other compartments of plant cells.¹⁵

When the ability of RootChip in live imaging of plant roots is integrated with advanced biosensors, the device can be used for real-time monitoring of free metals concentration in plant roots.¹⁶ Recently, the microfluidic-based RootChip and the genetically encoded Förster resonance energy transfer (FRET) sensor were integrated to investigate distribution and dynamics of cellular Zn²⁺ in plant cells for a better understanding of metal homeostasis signaling.¹⁷ This device overcomes 1) the limitations of local analysis methods such as imaging techniques of energy-dispersive X-ray or electron-spectroscopy with low imaging resolution¹⁶, 2) the constraint of radiation-induced X-ray fluorescence and particle-induced X-ray emission that can only perform static analysis of ions,¹⁸ and 3) the weakness of chemical probes in long-term imaging with limited control on intracellular localization of the probe.¹⁹

2.2 RootArray: Mapping genotypes to phenotypes in plants

To understand the complexity of gene regulation in multicellular organisms, the dynamics of gene expression needs to be quantitatively captured in both spatial and temporal aspects. To do so using conventional methods, the images captured from sample plant (shoot apical meristem)²⁰ at different angles were fused to achieve high resolution images. However, it was limited to one individual at a time with need to manual process for both imaging and data analysis. Microfluidics has shown the capability of long term imaging of a large number of specimens to assess spatiotemporal gene expression. RootArray is a higher throughput model of RootChip, developed to explore spatiotemporal gene expression dynamics in the roots of 64 *Arabidopsis thaliana* over several days. The device is made of a photopolymer scaffold and includes 64 wells filled with agar.²¹ When seeds are manually planted in all the wells and the chip is sealed, it is subjected to a bleach-containing solution, injected by a peristaltic pump for continuous exchange of different growth mediums. To initiate seed germination, the chip stands vertically for a few days until the roots penetrate the agar and reach the observation chambers. The chip is then returned to horizontal position for real-time monitoring and analysis of the roots growth (Fig 1b). The device is equipped with automated imaging systems for reconstructing 3D shape of each root and real-time screening of gene expression in individual cells. As part of the results, the expression patterns

of 12 transgenic reporter lines were recorded under four different growth conditions. The platform was used to assess the mapping of genotype to phenotype and to target the biological question of whether identical individuals under the same genetic condition represent alike phenotypes.

2.3 PlantChip: Phenotyping of the plant shoots and roots

Although RootChip was successfully tested for studying plants phenotypes, this device had the restriction of measuring phenotypes of only plant roots, while it was not feasible to quantify phenotypes of other plant organs.^{13, 14} PlantChip, a modified version of RootChip was recently developed for large-scale and quantitative analysis of the complete plant phenome of multiple *Arabidopsis thaliana* plants, not only at roots, but also shoots, hypocotyl, cotyledon, and even the a few leaves for a reasonably long period of time.²² The PlantChip is made of a series of funnel-shaped microchannels, where the seeds are automatically trapped at the narrow top opening of the funnel by hydrodynamic fluid loading. This hydrodynamic trapping avoids damage to the seeds during the positioning or manipulation. The roots elongate from the seeds through the seed holding site downward toward bottom site of the tapered microchannel (Fig 1c). In comparison with the RootChip, the observation chamber of the PlantChip is located in vertical position which enables continuous monitoring of whole organism. The performance of this device was tested by monitoring phenotypes of immutans mutant of *Arabidopsis*, and also recording the early invasion of zoospores pathogens to shoots and roots at different developmental stages. The device overcomes low temporal resolution of traditional plant phenotyping approaches and provides large-scale phenotyping of plants as a complement to genotyping analysis using genome-sequencing projects.²³ This platform also has high potential for early detecting the phenotypic interactions between pathogens and plant roots and shoots.

2.4 TipChip: Biological characterization of pollen tube growth

In addition to the applications of earlier microfluidic platforms for large-scale phenotyping, there are still several biological questions in the context of tip growing plant cells that need to be addressed, such as how a single site on cell surface can regulate the growth activity and how the dynamics of elongation is spatiotemporally controlled. The past methods were largely limited to phenotypes of mean growth rate and cell morphology, but for understanding the biology of complex process of tip growth, it was essential to employ complicated sensing methods to investigate several other parameters involved in the growth,

such as monitoring ion fluxes by vibrating probe, measuring the biomechanical properties of cell wall using a micro-indenter and quantifying the growth force by exposing the cell to mechanical sensors. These sensing methods are generally time consuming, suffer from variability and can only be used for one plant cell at a time. To overcome these constraints, TipChip experimental platform was developed with the ability of manipulating multiple tip-growing plant cells under precisely controlled condition, with the purpose of studying the biology of tip-growing plant cells.²⁴ TipChip, as an integrated microfluidic platform, was developed for characterizing the growth of tip growing plant cells with a focus on pollen tube. Pollen tube is the fastest tip growing plant cell that plays an essential role in life cycle of flowering plants. It germinates from pollen grain on stigma, senses multiple extracellular mechanical and chemical signals from its floral environment, and elongates in polarized shape toward ovule into interior of the flower. It has the function of delivering migrating sperm cells to eggs for fertilization purposes. Pollen tubes grow through different cellular tissues and follow extracellular guidance cues.

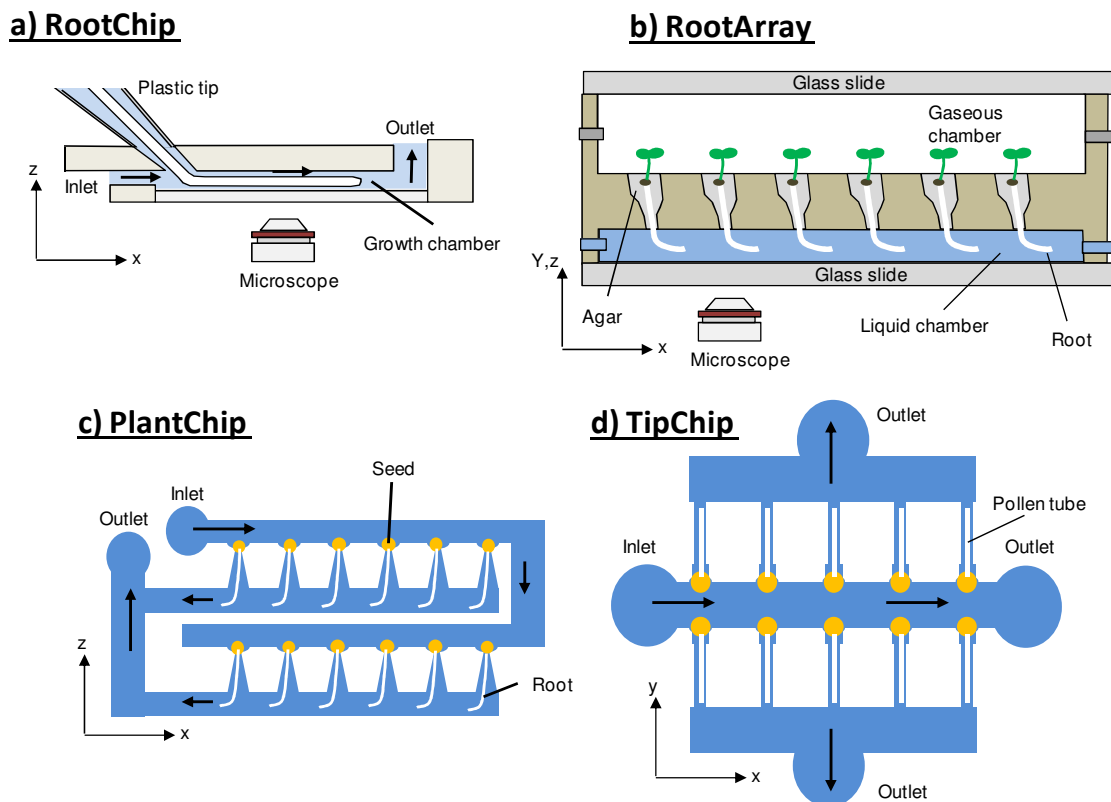


Figure 1 Microfluidic platforms for plant cells studies, **a)** The RootChip design. The seeds are manually located inside the plastic tips. The roots elongate through the agar-filled conical cylinders, reach the growth chamber and monitored

within the growth chamber in horizontal plane, **b)** The design of RootArray to study spatiotemporal gene expression dynamics in plant cells. Roots and Shoots grow in liquid chamber and gaseous chamber, respectively. The chip is sealed by two glass cover slips at both top and bottom surface. The roots can be monitored in both vertical and horizontal positions, **c)** Schematic design of PlantChip for high-throughput plant phenotyping. Seeds are hydrodynamically trapped at the entrance of the funnels. The roots and shoots are monitored in their vertical positions, **d)** The overall design of TipChip with serially arranged growth microchannels. The pollen tubes are automatically trapped at the entrance of microchannels. The growth of pollen tubes are monitored along the growth microchannels in horizontal plane.

From design perspective, the TipChip is made of two PDMS layers permanently bonded under plasma activation, where the bottom layer consists of a series of growth microchannels connected to fluidic inlet and outlets to allow elongation of individual pollen tubes through microchannels. Two main medium outlets are responsible for conducting some pollen grains toward the entrance of microchannels and directing the leftover pollen grains toward outlet drain.²⁴ The pollen tubes start germination from trapped pollen grains, followed by elongation along growth microchannels in horizontal plane (Fig 1d). This platform was tested to study growth behavior of *Camellia* pollen tube. The results showed that both germination rate and growth rate of pollen tubes within TipChip are comparable with the results obtained from conventional *in-vitro* assays. This confirms that the TipChip can provide a suitable environment for studying serially arranged tip growing pollen tubes with the purpose of targeting several unknown biological questions.

The results of exposing pollen tubes to the flow of medium show that the growth rate is very sensitive to the fluid velocity and thus, excess flow velocity may arrest the tube growth, cause cell bursting, and crucial for phenotypic analysis.²⁴ For this purpose, the dimensions of the TipChip were optimized in order to ensure the entrapment of only one or two pollen tube at the entrance of each microchannel, and to provide identical growth condition along all microchannels as prerequisite for further chemical simulation or toxicity testing on plant cells.²⁵ Although this platform was used for pollen tube it can be applied for fungal hyphae, root hair, and some other tip growing plant cells.²⁶ Due to the structure of serially arranged microchannels, different biosensors such as microgaps or microcantilevers were integrated along the microchannels to detect the response of plant cells to external chemical or mechanical stimulations as discussed in sections 3,4.²⁷

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In another study, a design similar to the Tipchip was fabricated to characterize the growth of *T. fournieri* pollen tube with smaller diameter than the *Camellia* species tested in TipChip.⁷ The effect of microchannel dimensions on behavior of elongating pollen tubes was studied. The dimensions of microchannels were confined to a size comparable with the pollen tube diameter in order to enable observation of growing tube in a single focal plane and accurate growth analysis. They found that the width of microchannels did not influence the growth rate of pollen tube, but there was an optimal value of 8-10 μm for the height of microchannel for *T. fournieri* pollen tube.⁷

3 Chemical stimulation of plant cells within microfluidic environment

Beyond characterizing plant cells growth within microfluidic environment under regular condition of medium, the advantage of rapid and accurate exchange of growth medium allows to study the cell response to environmental changes, toxic agents or mutation alteration within microfluidics. By changing the growth medium, either globally or locally around the plant cells, different cellular phenotypes such as change of growth rate or growth orientation, alteration of gene expression, cell bursting or cell death are exploited to characterize response of plant cells to external chemical cues.

3.1 Global treatment of plant cells within microfluidics

3.1.1 Microfluidic environment for studying toxicity effects on plants

In last decades, the influence of environmental pollutants and toxic agents on plant viability has gained significant importance, however accurate determination is a crucial task. The large variations in response patterns of different plant species to pollutants resulted in presenting different values for their half maximal effective concentration (EC 50). Moreover, due to exposure of plants to several pollutants, characterizing the effect of a single substance, independent of other agents is challenging. The conventional laboratory-based techniques have not been able to conquer these challenges, since these techniques usually need a large number of single tests and large volume of toxic agents. The development of high-throughput platforms for exploring multidimensional influences of environmental agents on plants is vastly promising. Microfluidics has proven its strength to rapidly and stably change the growth medium in nanoliter scale with high-throughput potential, for applications in drug development and cellular studies. When the growth medium is changed globally, the whole medium within the fluidic network is replaced with a new medium containing a chemical or toxic agents or a drug.

The effect of toxic agents on plant cells and several microorganisms has been effectively investigated within microfluidic environment using globally change of growth medium. Green algae *Chlorella vulgaris*, as one of the main producers of atmospheric oxygen is very vital for the ecosystem. Different pollutants such as herbicides and chemicals containing heavy metals can alter the amount of these algae and, as a result, the oxygen supply of the ecosystem. To effectively screen the effect of toxic agents on algae *Chlorella vulgaris*, they were cultivated in a microfluidic device and the dose dependent analysis of sample algacide CuCl_2 agent on algae was implemented on 350 fluid segments. The half maximal effective concentration (EC 50) of CuCl_2 on green algae C was measured by photometric characterization in a microfluidic device. The results were comparable with the labor experiments obtained from microtiter plates.²⁸

Plant roots are the sensitive compartments of plants that have been used to detect the presence of pollutants in the environment. RootChip and RootArray were successfully tested to characterize the change of root growth under the change of growth microenvironment.^{13, 21} The roots were exposed to the Gal sugars known as root growth inhibitors. The exposure of roots to the Gal sucrose caused darkening of the tissue detected by Bright-field microscopy, while the epifluorescence analysis revealed the disruption of normal cytosolic signal as a sign of cell death.¹³ Pollen tubes, other sensitive compartments of plants, have been extensively used in open assays to detect the presence of different pollutants on plant viability. Pollen tube is extremely sensitive to external chemical signals, which makes it a suitable cellular model for characterizing cell response to the influence of environmental chemicals as well as various agents involved in the cellular regulatory mechanism.^{29, 30} The results obtained from toxicity testing on pollen tubes was verified with the response of animals to toxic materials, which indicated that the study of pollen tube behavior will have the potential of detecting toxic agents even on humans.³¹ The effect of toxic substances, detergents, pollutants, and metal ions on pollen tube growth was investigated by measuring parameters such as percentage of pollen grain germination, change of growth rate and alteration of pollen tube morphology.³²⁻³⁶ In order to provide common understanding from the potential cytotoxic degree of test substances, the Pollen Tube Growth (PTG) protocol introduced the C50 criterion.³⁷ We used our enhanced microfluidic device to integrate a large number of individual pollen tubes as individual whole cell sensors to capture the growth with high resolution images. The device was

used to detect sucrose concentration as one of the main agents involved in regulating turgor pressure. Moreover, the dose-dependent toxicity of aluminum ions on growth behavior of pollen tube was detected with a higher accuracy respect to the previous open assays.³⁸ The enhanced design had been optimized to afford stable environment for changing the growth condition. The parameters of growth rate and cell bursting were used to detect the concentration of aluminum ions.

3.1.2 Microfluidics for detailed characterizing dynamic growth of tip growing plant cells

Tip growing plant cells do not elongate in a steady manner, but display an oscillatory dynamic growth. This can be considered as one common characteristics of extension in these cells.³⁸⁻⁴⁰ The oscillatory behavior has impact on growth regulatory mechanism and it is correlated with oscillatory behavior of several other parameters involved in regulation of cell growth.^{40, 41} The growth oscillation is subjected to modulation in response to external triggers. The temporal and spatial resolution of previous open assay experiments hindered accurate characterization of oscillatory growth pattern. These experiments had mainly focused to measure variation of average growth rate, oscillatory period, and oscillation amplitude under chemical stimulation.⁴¹⁻⁴³ A few researches studied the parameter of frequency of oscillation as the dynamic feature of pollen tubes.⁴¹ The primary peak oscillation frequency of pollen tube and its variation under chemical treatment has been detected in open assays. To overcome the limitation of detecting higher mode oscillation frequencies, we used the modified TipChip design for high resolution imaging of the *Camellia* pollen tube growth elongating in liquid medium without their displacement during the imaging process.³⁸ Using this platform, the existence of both primary and secondary peak frequencies and their variations under the effect of different sucrose concentrations were detected. It was also illustrated that even in apparent absence of low frequency oscillations in young pollen tubes, the secondary peak frequencies always exist which indicates that pollen tube has always a dynamic growth. Both primary and higher mode oscillatory frequencies were shifted under the sucrose treatment, indicating how pollen tube regulates its growth at different modes of oscillation (Fig 2). The relation of these frequencies to other physiological processes, such as oscillation of extracellular and intracellular ion gradients and ion fluxes can be further studied.^{41, 42}

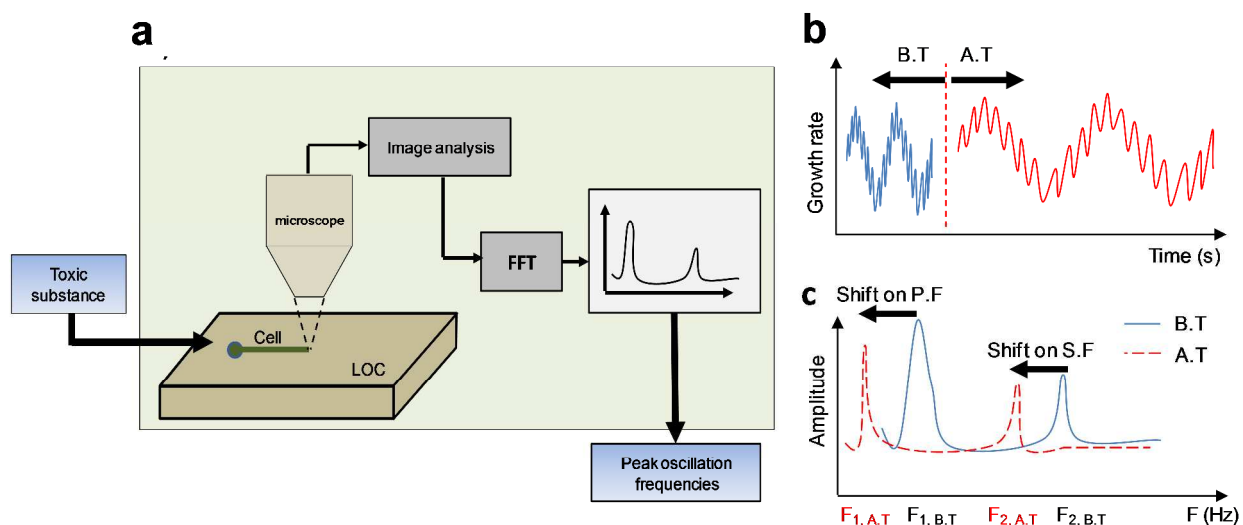


Figure 2 Whole cell toxicity sensor, **a**) Experimental setup for detecting both primary (P.F) and secondary (S.F) oscillation frequencies of pollen tube growth rate, **b**) Primary and secondary frequencies are shifted under chemical treatment on pollen tube's apex. $F_{1,B.T}$ is the primary frequency of oscillation before treatment. $F_{2,B.T}$ is the secondary frequency before treatment. $F_{1,A.T}$ is the primary frequency after treatment. $F_{2,A.T}$ is the secondary frequency after treatment.

3.1.3 Microfluidics for accurately detecting the change of gene expression and its relevance to cellular phenotyping

To realize the influence of chemical environment on gene expression in plants, the RootArray investigated the time-dependent expression alteration of WOX5 and UPB1 lines, as known agents with significant expression changes. The progressive changes were detected for both UPB1 and WOX5 expression in low pH in the meristem and elongation zones. The expansion of GFP expression from cell to cell was also detected and self-sustaining regulatory network controlling WOX5 expression was identified.²¹

3.2 Local treatment to reveal aspects of cellular mechanisms of plant cell growth

In addition to the aforementioned applications of rapid and reliable change of growth medium, globally around growing plant cells, local treatment of tip growing plant cells can help to understand several other aspects of plant cell biology. Here, we explain how local targeting of elongating pollen tubes and plant roots explores the growth mechanism of these cells.

3.2.1 Reorientation of pollen tube growth in response to local chemical gradient

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The pollen tube is a very sensitive plant cell model to chemical signals, as it responds to these triggers by changing its elongation rate or growth direction.^{44, 45} This sensitivity is required for pollen tube to perform its main function to carry sperm cells through several tissues and to deliver them to ovule.⁴⁶ It is commonly agreed that the growth activities of pollen tube is confined to its tip, where the intracellular transport and signaling mechanisms are regulated to maintain polar growth.⁴⁷⁻⁵¹ Pollen tube has symmetrical growth under analogous growth conditions around its apical domain, but exposure of pollen tube to local asymmetric triggers can disrupt it. These local cues can be generated by ovule in form of electrical signals or chemical gradients attracting or repelling the pollen tube.^{52, 53} There is still a great biological interest to realize what agents are involved in reorienting growth direction, either attractive or repulsive, and how the cellular mechanism of growth is regulated by external chemical signals to force growth redirection. Developing new techniques for precise manipulation of elongating pollen tube at subcellular resolution is very desired to locally apply different concentrations of growth inhibitors or promoter agents to growing site of plant cells.

Microfluidics has been utilized for *in-vitro* study of pollen tube redirection toward ovule.⁵² A microsystem-based assay was developed to mimic the microenvironment of ovule fertilization and to model the redirection of *Arabidopsis thaliana* pollen tube in response to chemoattractants secreted by the unfertilized ovules. In this device, pollinated pistils are placed within microgrooves, while pollen tubes elongate from pistils and grow through microchannels toward two side chambers. One of the side chambers is occupied by ovules while the opposite chamber is left empty. This generates asymmetric concentration around pollen tube's tip. The reorientation of pollen tubes growth direction toward ovules suggests that they sense a concentration gradient from the ovules site (Fig 3a).⁵²

Although embedded ovules placed within micro-chamber proved the presence of plausibly a chemical signal from ovules toward pollen tubes, the type of chemical agent and its concentration gradient is difficult to detect. As an alternative method, a local treatment at the subcellular resolution can generate an accurate chemical gradient over growing pollen tube. Local micropipetting was applied to elongating plant cells, where chemical agent was locally introduced to cellular region of interest.^{54, 55} Despite its relative success, the micropipette handling needs a manipulation mechanism that makes it a time-consuming

process. Moreover, the eventual concentration of chemical agent at the vicinity of target region is not fully controllable due to presence of both diffusion and convection. To overcome this challenge, microfluidics has been used to provide precise local concentration of agents. Due to small Reynolds number ($Re < 1$) in microchannels and trivial convection in microfluidic, the liquid flows can be mixed only by molecular diffusion and this provide stable laminar flows along the microchannel. ⁵⁶ Depending upon the flow velocity and the diffusion coefficient, the interface between two streams can be adjusted to target different regions of growing plant cell. Local chemical administration to pollen tube at subcellular resolution has successfully been implemented using laminar flows in Tipchip microfluidic device (Fig 3b). ²⁴ Additional to the laminar flow-based gradients, the diffusive gradient was recently exploited to expose pollen tube of *Torenia Fournieri* to concentration gradient of a signaling molecule, LURE peptide, in order to study the pollen tube guidance quantitatively. ⁵⁷ The results show that pollen tube within the reaction chamber was guided toward higher LURE concentration (Fig 3c).

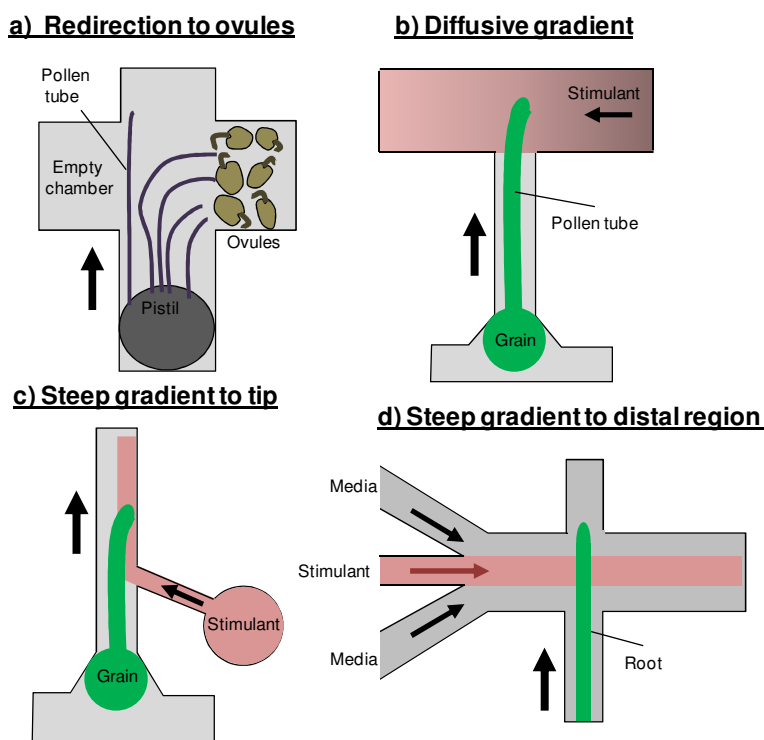


Figure 3 On-chip chemical stimulations performed on tip growing plant cells in spatial resolution, **a)** Ovules positioned within a chamber attract pollen tubes, **b)** Diffusive gradient in microfluidics for stimulating pollen tube's tip, **c)** Steep gradient in microfluidics to target pollen tube's tip, **d)** Steep gradient in microfluidic using multi-laminar flows for local treatment of growing root to its distal region.

3.2.3 Monitoring the transport mechanisms of auxin in *Arabidopsis thaliana* root using multi-laminar flows within a microfluidic chip

Plant roots with the main function of water and nutrient uptake respond to the external environmental cues and regulate their plasticity at different developmental stage. To realize the interplay between root and its microenvironment, the response of cells within root to the external signals needs to be detected, which requires high spatial and temporal resolution of manipulation and detection. However, the conventional methods are limited to spatial control in the order of millimeter.⁵⁸ In addition, when the medium is altered on agar plates, the chemical gradient around roots has low stability and cannot be accurately quantified. Microfluidics can help to better understand the mechanism of root development by local manipulation of elongating roots to different chemicals such as nitrogen, phosphate, salts, and other hormones. The roots of *Arabidopsis thaliana* plant were exposed to local chemical gradient within microfluidic environment (Fig 3d).⁵⁹ The multi-laminar flows in microchannels provided a high spatial resolution of flows toward the live roots. The flows were optimized to ensure that shear forces don't affect root growth. The auxin derivative, 2,4-dichlorophenoxyacetic acid (2,4-D), and its inhibitor N-1-naphthylphthalamic acid (NPA) were examined at a spatial resolution to differentiate the active and passive transport mechanisms of auxin through observing the level of GFP expression associated with the position of auxin. It was also proved that 2,4-D local stimulation improves the morphological changes of epidermal hair growth.⁵⁹

4 Microfluidics to study plant cells biomechanics

4.1 Bending-Chip: Quantification of mechanical properties of plant cell wall

Modeling plant cells growth essentially needs quantitative value for mechanical properties of cell wall. Plant cells, in comparison with animal cells, are surrounded by a stiff cell wall made of polysaccharides. Due to microscopic size of individual plant cells, direct measurement of mechanical properties of cell wall has been challenging^{58, 59}. Indirect methods of tensile testing on plant tissues, measured by instron instrument, estimate mechanical properties of plant cell walls.⁶⁰ Pressure probe as another effective technique increases internal turgor pressure by injecting oil into the cell, followed by measuring the volumetric Young's modulus of cell wall.^{61, 62} These techniques have been used to roughly estimate the mechanical properties of cell walls, but they are still indirect methods and not accurate for

characterization of subcellular resolution of cell wall. To directly target cell wall mechanical properties, micro-indentation and atomic force microscopy have been exploited for plants studies, where local compression force is applied on cell wall.⁶³ However, due to local manipulation, the estimated value for Young's modulus may not reasonably correspond anisotropic cell wall made of a complex composition of multiple polysaccharides.⁶⁴⁻⁶⁶ Mechanically speaking, to measure tensile strength of plant cell wall, the cell should be subjected to tensile or bending loads which is technically challenging due to the small size of most plant cells.⁶⁷

Recently, a microfluidic device, called Bending-Lab-On-a-Chip (BLOC), was developed to implement bending test on small plant cells to quantify the Young's modulus of cell wall. The design of BLOC is similar to the Tipchip. Pollen grain suspension is introduced into chip and pollen tube elongating along the growth microchannel is exposed to a bending fluid loading. The Young's modulus of pollen tube cell was estimated by measuring pollen tube deflection and exploiting a numerical model. This value was in agreement with the results of pressure probes and the values estimated from the testing on the reconstituted cellulose-callose material as the main polysaccharides of pollen tube cell wall.⁶⁸ This technique is a direct method for measuring elastic modulus of cell wall and has the advantage of presenting a more global value for the mechanical properties of cell wall, independent of its local anisotropies.

4.2 Microgaps for quantification of growth force in tip growing plant cells

In addition to biomechanical contribution of microfluidics in determining mechanical properties of plant cell wall, microfluidics has also been used to investigate interaction of plant cells and their surrounding tissues in order to better understand invasive lifestyle of plant cells.⁶⁹ Plant cells elongate through the extracellular tissue or matrix to perform their biological task, however, the cellular mechanism involved in the invasion to the tissues has not been fully explored. The plant cells are hypothesized to use two strategies in combination to invade their extracellular matrix: 1) generating enzymes locally to soften the tissue or matrix at the growing site, 2) exerting invasive force to deform the tissue.^{70, 71} The driving force required for both elongation and invasion is generated from the internal turgor pressure. Only a portion of the turgor pressure can be exerted to its surrounding tissue (invasive force) and significant part of driving

force is used to overcome cell wall stiffness and to yield the cell wall for continuous elongation.^{72, 73} Agarose-stiffened growth matrix has been used to qualitatively determine capability of plant cell for invasion, but obtaining quantitative values for the invasive force requires integrating sensors in subcellular size, which is difficult to make.⁷⁴ Conventional strain gauge was placed perpendicular to growth direction of fungal hyphae in order to quantify the growth force. However, due to change of cellular shape under the contact with gauge sensor and redirection of growth, the measured values were not conclusive.⁷⁵

We have recently developed a microfluidic device to investigate the penetrative force of pollen tube.²⁷ In this regard, the TipChip design was adapted by incorporating mechanical constricts along the growth microchannels in order to provide obstacles against the growth. The individual pollen tubes elongating through the microchannels interact with the polydimethylsiloxane (PDMS) microgaps which resembles the apoplast of transmitting tissue. The results of interaction of pollen tubes with microgaps show that pollen tubes are able to exert their penetrative force (wedge force) to the gaps and deform these micro-constricts (Fig 4a). It was also observed that some of pollen tubes burst during the interaction with PDMS obstacles, which raised the argument that pollen tubes regulate their cell wall mechanical properties (softening cell wall at the apex) in order to generate sufficient force to pass through the gaps.²⁷ Since the microgaps push the pollen tube to pass through the gaps, the force measured through the interaction of microgaps and pollen tube is more conclusive than the value obtained from gauge sensor testing, where the growth was redirected during the interaction with the mechanical sensor.

5 Other applications of microfluidics for plant cell science

In addition to the current development of diverse microfluidic devices for characterizing plant cells behavior under the influence of in-vivo-like chemical or mechanical cues, microfluidics have also been developed for several other aspects of plant cell biology.

5.1 Presence or absence of directional memory in plant cells

The growth direction of tip growing plant cells is controlled by the cellular regulatory mechanism at the starting point of elongation, the external triggers at the growth site, or both. The "inner memory" is a term used to explain whether the growth direction of tip growing cell is dependent on the position of starting point of elongation. Such behavior has been observed in root hairs and fungal hyphae.⁷⁶ For plant root

hairs, the main function is to uptake sufficient water and nutrients. To perform the function most efficiently, the root hairs should be able to continue the growth perpendicular to the roots, while they navigate through several obstacles. Under both chemical and mechanical stimulations, the growth direction returned to its normal growth away from the root.⁷⁶

Systematic analysis of "inner memory" in plants requires subcellular microenvironment under which the *in-vitro* growth pattern of the plant cells is investigated. The conventional methods lack the ability to study the presence or absence of "inner memory" on tip growing plant cells. Microfluidic devices instead provided such microenvironment to test the directional memory of fungal mycelium.⁷⁷ The cells were exposed to purposefully designed microstructures mimicking fungi's natural environments. The results showed that fungi use space-searching strategies to control growth direction through which it does not exclusively rely on wall geometry.⁷⁷ The presence of directional memory is necessary for these cells to perform their function efficiently. To know whether other plant cells have directional memory or not, a microfluidic device recently investigated the presence of directional memory for pollen tube, where pollen tubes were grown along serpentine-like microchannels. The tubes slide along the microchannel wall and only changed their growth direction when they touched the wall, meaning that in absence of channel wall, the tubes maintained their last growth direction. In addition, after elongation through repeated turns in the microchannel, the tubes were exposed to a wide chamber giving a chance to choose the growth direction. The results show that the tubes continued elongation based on their last growth direction at the microchannel exit and independent from their initial growth direction germinated from pollen grains. This behavior is consistent with the pollen tube mission, where its growth direction toward ovule should be conducted by external guidance cues and not necessarily by position of pollen grain.

5.2 Plant cell division in microfluidic environment

Traditional plant cell culture needs large volumes of media and storage. In this context, a microfluidic device can provide a platform that uses a smaller cell population size and controllable flow of culture medium. Microfluidics has been utilized to culture plant cells with the purpose of engineering these cells.⁷⁸ The protoplasts of *Nicotiana tabacum* were cultured in microchannels in Nitsch medium. The cells were divided and micro-colonies were formed within 4 weeks with no damage to the plant cells in culture. The

results show that cell division had occurred 3 days earlier in PDMS channel in comparison with the ordinary culturing in open assays.⁷⁸ Due to highly spatiotemporal control of microenvironment and ability of supplying optimal supplementation of nutrients, the growth continued until visible cell mass was formed in microfluidic environment.⁷⁹ The recent progress in on-chip protoplast culturing also resulted in 85% improvement in the percentage of first protoplast division in microfluidic environment.⁸⁰ The long-term culture of plant cells in microfluidics is promising for further biological analysis and eventually to develop *in-vitro* plant disease models.

5.3 On-chip electrofusion of plant protoplasts

Last but not least, microfluidics has the application for study of plant cells fusion within *in-vitro* environment, which is important to be characterized for better understanding of cell characteristics. In plants, the fusion of protoplasts from different species enables studying genetic modification and plant breeding⁸¹. The cell fusion mechanism is known as the generation of a little pore on the membrane of two protoplasts, followed by their fusion to form a single cell under the effect of surface tension. Among different techniques for *in-vitro* fusions, electrofusion has been used for fusion of different cell types as well as plant cells, and has the advantage of being a secure fusion process for fragile protoplasts.⁸² However, conventional electrofusion uses a large number of cells due to lack of a control system to manipulate individual cells. The integrated microfluidics device was developed to implement electrofusion process at single cell level with a superior control on cell manipulation. For plant cells, a microfluidic device was designed to perform electrofusion process on five different plants (*Arabidopsis thaliana*, *Nicotiana tabacum*, *Peucedanum japonicum*, *Glehnia littoralis* and *Brassica campestris*).⁸³ The design of this electrofusion chip consists of the fusion chamber, the inlet-outlet ports, the cell delivery channel for manipulating the cells, and the microelectrodes to provide electrical power required for electrofusion (Fig 4b). This microfluidic device reported a cost-effective cell and nutrient delivery and a successful electrofusion system with the application in gene transfection studies.

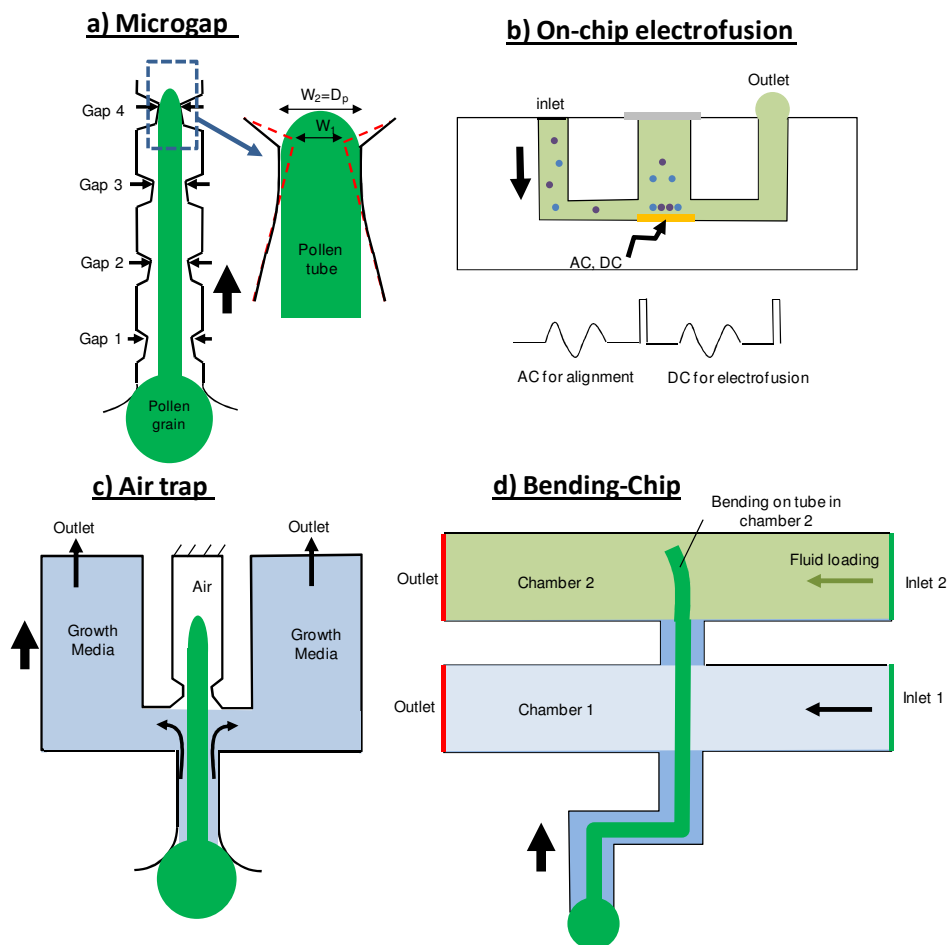


Figure 4 Applications of microfluidics to study plant cell biomechanics, electrofusion and biological studies, **a)** A series of PDMS microgaps integrated along microchannel for quantification of pollen tube growth force. W_1 is the initial position of microgap, W_2 is the position of microgap in deformed position, **b)** On-chip electrofusion of plant cells. Combination of AC and DC pulses for alignment and electrofusion of the protoplasts, **c)** A schematic of air trap design for local stimulation of pollen tube's distal region, **d)** A sequential bending on growing pollen tube within the chambers to study the influence of drug or toxic agents on mechanical properties of cell wall.

5.4 Microfluidic-based point-of-diagnosis device for detecting plant cells deficit

Apart from applications of microfluidics for plant cells biology, they can be used to improve plant reproduction. The assessment of plant grains quality after harvest can be mostly performed using visual inspection, however for some plant defects such as sulfur deficiency, more advanced techniques are needed. Many countries are suffering from low-grain sulfur content as the cause of poor dough and baking quality, hence developing new techniques for detecting convenient and simple ways of analysis is

highly demanded. In this regard, an industrialized electrophoresis-based microfluidic chip was developed to detect sulfur content of grains, where gliadin proteins together with water-soluble proteins were extracted from ground grain and applied directly to a Protein 230 chip for analysis on the Bioanalyzer 2100 Lab-on-a-chip equipment in order to detect the area of profile containing ω -gliadin.⁸⁴

6 Challenges and future research on plant cells using microfluidics

Despite all aforementioned microfluidic platforms developed for plant cell studies, the progress of microfluidics for studying plant cells biology is not comparable with the applications of microfluidic developed for animal cells. Several platforms proved their capability for plant root studies, however more progress is expected in different aspects of plant root growth such as colocalization studies, mutant screening using fluorescent markers, variation in natural populations, and high-throughput determination of the growth characteristics using different cell wall dyes. These platforms will have the ultimate goal of developing effective approaches to produce more crops that clearly requires better understanding of how different nutrients are taken up by roots and how these roots interact with pathogens.

Local treatment of plant cells will help us to answer some biological questions such as how water uptake and ion fluxes through not the apical region, but the distal region of plant cells may regulate growth mechanism. One of the main challenges of conventional methods is the lack of local control to change culture medium only at the distal region of plant cells or at the apex, any change of culture medium influences both the distal region and the tip. This made it difficult to distinguish contribution of tip or distal region on the growth behavior. Understanding the effect of ion fluxes or water uptake through distal region requires novel microstructures within microfluidic devices. A novel *air trap* microstructure was tested in such a way that liquid medium surrounded only the distal region of pollen tubes without any influence on the tip trapped in air chamber (Fig 4c).²⁴ Such platform will have high potential for answering a number of biological questions related to the role of diverse ion gradients at the apex. It may also reveal new clues about the role of internal ion stores such as Ca^{+2} stores in adjusting calcium gradient at the apex.⁴¹ A number of molecular cues were nominated to be effective on pollen tube reorientation. They can be locally exposed to pollen tubes within microfluidic environment to characterize pollen tube guidance to or repulsion from a female gametophyte.

Characterization of cell wall mechanical properties helps to determine contribution of cell wall to growth behavior and significantly benefits our understanding of plant cell biomechanics. The Bending-Chip was formerly implemented to quantify the Young's modulus of pollen tube cell wall via bending test on growing pollen tubes.⁶⁸ However, the challenge still remains to quantify the alteration of cell wall mechanical properties under the effect of drug or toxic agents, which has always been under interest of biologists. The modified Bending-Chip will hopefully allow to quantify the effect of enzymatic treatment on alteration of cell wall elastic modulus. The plant cell will be subjected to bending fluid loading in subsequent testing chambers, where each chamber contains a portion of pollen tube exposed differently to a drug or toxic agent (Fig 4d).

Finally, several other aspects of invasive lifestyle of plant cells still remain to be discovered. One is to explore the impact of cytoskeleton in generating invasion force in tip growing plant cells.²⁷ The cytoskeleton-based force was shown trivial in comparison with turgor pressure, but some cues exist against this general understanding of invasive mechanism.⁸⁵⁻⁹⁰ For instance, lower actin depolymerizing drugs play role to invade an agarose-stiffened substrate or some fungi with invasive lifestyle have shown F-actin depleted zone.^{91, 92} There are several other parameters involved in the invasive mechanism of tip growing cells such as 1) the effect of friction force between growing tip and extracellular matrix, 2) digestion of extracellular matrix by enzymatic activities or other biochemical means,^{85, 89} and 3) modulation of cell wall properties under local mechanical and chemical stimulation during invasion to tissues. All these questions are expected to be addressed by developing novel microfluidic platforms. Integrating new synthesized polymers and tissues into microfluidics with different frictional and mechanical properties for adjusting friction force between the tip and the extracellular matrix will be beneficial to determine contribution of friction in invasive force. In this context, influence of several proteins produced by pollen tube or pistil such as expansins⁹³⁻⁹⁵, cutinase⁹⁶, polygalacturonase⁹⁷, endoxylanase⁹⁸, pectinase⁹⁹, pectin esterase¹⁰⁰ and pectin methyl esterase^{101, 102} can be evaluated. Several techniques earlier developed using microfluidics to characterize traction force in animal cells such as arrays of elastomeric micro-pillars or beads-embedded elastomer as the force sensors. These techniques can be exploited to study the invasion in plant cells.^{103-104, 105}

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