Plant Protoplast scRNA-Seq (ppRNA-Seq) on the Nadia instrument

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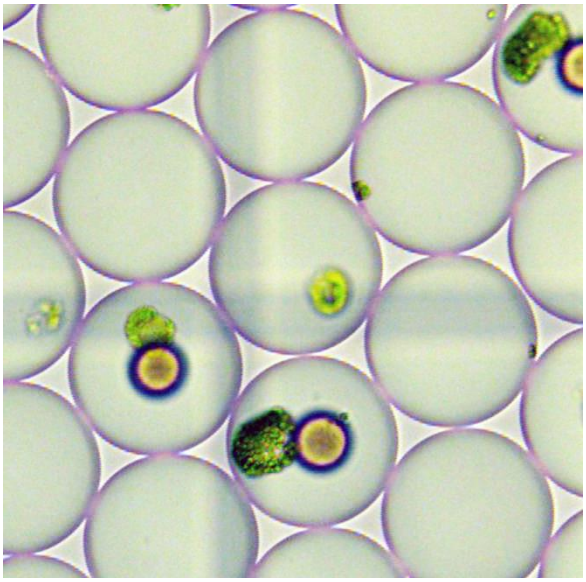

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Summary

This application note explains how the Nadia Innovate can be used to encapsulate plant protoplast cells in high viscosity buffers ready for single cell RNA sequencing. We demonstrate that this standardised single cell protocol can assay tens of thousands of isolated plant leaf cells, yielding high quality transcriptomic data from varied leaf cell types. Methodological advances such as this could accelerate the progress of plant leaf research in the single cell field.

Introduction

Large-scale transcriptomic studies of single protoplast cells from plant tissues have generally lagged behind those of animal models. Unlike many mammalian cell types, which dissociate from each other more readily, plant tissues require intense enzymatic digestion and specific buffer conditions to be accessible for conventional single cell and microfluidic techniques.

The most prolific model system within plant genomics is *Arabidopsis thaliana*, thanks to its well-characterised genome, experimental amenability and applicability to other plant systems [1]. A wealth of single cell studies have been conducted on the root cells of *A.thaliana* [2–5]. However, large scale transcriptomic datasets of single leaf cells are remarkably absent [6]. Where single cell leaf transcriptomes are available, isolation is conducted with microdissection [7], limiting potential throughput. This disparity is primarily due to technological challenges.

Plant root cells have inherently been more amenable to single cell workflows than those of other plant tissues, due to both their relatively uniform size (often not exceeding 40um) and ease of harvesting [5].

By contrast, plant leaf cells, such as those within the mesophyll layers, can be anywhere between 10um and 60um within a single sample [1,8], preventing them from being encapsulated by the narrow (typically only 30 µm) junctions of some microfluidic platforms [9]. Plant leaf tissue contains varied and precisely arranged cell types which represent tempting subjects for high-throughput single cell sequencing. Single cell transcriptomic studies of leaf cells can reveal important aspects of plant and crop biology which root cells cannot, such as insight into photosynthetic pathways and gaseous exchange [7].

Once plant protoplasts are isolated, they must be stored in buffers with similarly high osmolarity to avoid osmolysis [10] and stress responses in measurable transcriptome states [11]. Buffers of the complexity necessary to maintain plant protoplast viability are typically much thicker and adherent than animal cell buffers used in microfluidic processes common to droplet-based scRNA-Seq protocols [12,13]. This added viscosity is another technological challenge facing high throughput microfluidic solutions in plant research, requiring variable pressure to be applied to each buffer to account for different flow rates.

Here we demonstrate that the Nadia Innovate and Nadia Instruments are capable of efficient and high-throughput encapsulation of *A.thaliana* leaf protoplasts in multiple buffers tailored to plant protoplast integrity. Furthermore, we show that the integrated single cell RNA-sequencing workflow for use with plant protoplasts (ppRNA-Seq) developed by Dolomite Bio generates high quality transcriptomic data from both large and small plant leaf protoplasts.

Material and Methods

Droplet system

Dolomite Bio's Nadia Instrument is designed to allow high-throughput analysis of single cells and single nuclei using droplet microfluidics. It produces highly monodisperse droplets using three independently controllable pressure pumps.

With the addition of the Nadia Innovate module (Figure 1), the Nadia Instrument is converted into an open development platform for the optimisation of new applications and protocols. Customisation options allow the dropletization of diverse cell types in both high viscosity and high complexity buffers.

Once pressure profiles for generating droplets are developed on the Nadia Innovate, the parameters are be transferred to the Nadia Instrument to be run in high throughput. The chips used in this application note feature fluorophilic 80 µm microfluidic junctions capable of encapsulating cells or objects up to 60 µm in diameter.

Figure 1: The Nadia Instrument (right) and the Nadia Innovate platform (left) set up depicting all the components of a Nadia Innovate system.

Plant culture conditions (*Arabidopsis thaliana*).

Arabidopsis thaliana seedlings were grown according to Yoo et al., 2007 [14]. At 4 weeks of age, healthy leaves were removed, cut into 1 mm strips using a clean sharp blade and digested in Cellulase R10 and Macerozyme R10 enzyme solution for 4 hours as described in Yoo et al., 2007 [14]. Once digested, protoplasts were resuspended and stored in either WI [14] or W5-$CaCl_2$ buffer (Adapted from [15]) at a concentration of 300,000 cells/ml. The compositions of these specialised plant-optimised buffers are detailed in Table 1.

Table 1 *Composition of two high-viscosity buffers optimised to maintain integrity of plant protoplasts*

	WI buffer [14]	**W5-$CaCl_2$** Adapted from [15]
Component	**Concentration (mM)**	
MES (pH 5.7)	4	2
Mannitol	500	-
KCl	20	5
NaCl	-	154

Following resuspension, plant protoplasts were observed under a light microscope to assess their level of debris and viability. *A.thaliana* leaf protoplasts displayed little sign of osmolysis and minimal debris in both WI and W5-$CaCl_2$ plant-optimised buffers (Figure 2).

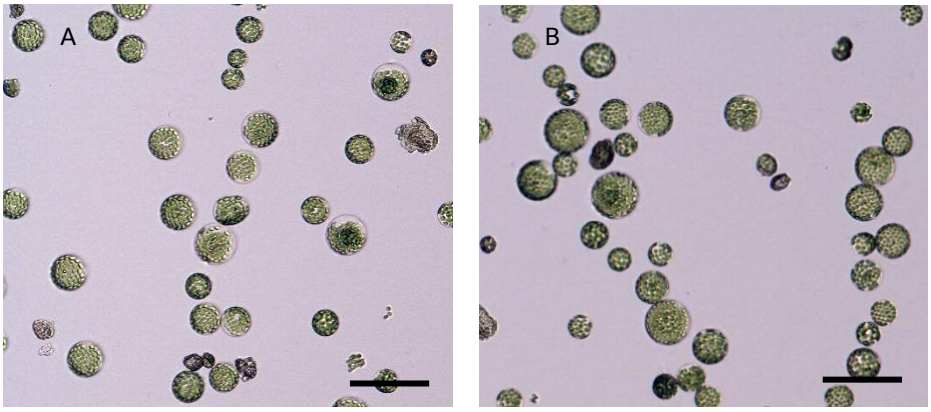

Figure 2 *Isolated A.thaliana leaf mesophyll protoplasts resuspended in WI (A) and W5-$CaCl_2$ (B) buffers. Scale bars represent 100 µm.*

Plant leaf protoplast encapsulation on the Nadia Instrument

Flow rates for oil, each of the high viscosity plant cell buffers (Table 1) and lysis buffer for use in single cell plant protoplast scRNA-Seq were optimised using the Nadia Innovate platform. By altering the pressure levels in real time and observing their effects on droplet formation using the high-speed digital microscope of the Nadia Innovate, novel pressure profiles were generated to form consistent droplets with these high complexity buffers. Once optimised, the novel protocol, ppRNA-Seq, was ported onto the Nadia platform to be run as a standard protocol with multiple samples.

Single plant protoplast RNA-Sequencing on Nadia (ppRNA-Seq)

Isolated *A.thaliana* protoplasts in both WI and W5-$CaCl_2$ buffers were encapsulated alongside barcoded oligo-dT capture beads suspended in lysis buffer using the novel ppRNA-Seq protocol on Nadia. Composition of the lysis buffer and barcoding of the capture beads adhered to the established Drop-Seq protocol [13]. The period of lysis following encapsulation was increased from 10 minutes to 20 minutes. Following lysis, droplets containing sample mRNA bound

to oligo capture beads underwent emulsion breakage, reverse transcription and PCR amplification (15 cycles) into cDNA libraries as per the scRNA-Seq on Nadia protocol. cDNA libraries were produced from a subsample of the transcriptome-bound beads from each sample. Libraries for each sample were constructed from a total of 5000 beads, which at the initial cell and bead concentrations should yield ~250 cell transcriptomes [13].

For the full detailed ppRNA-Seq protocol, see the Plant Protoplast scRNA-Seq (ppRNA-Seq) on Nadia protocol available at https://www.dolomite-bio.com/support/downloads/

Next generation sequencing and bioinformatic analysis

cDNA libraries were tagmented using a Nextera XT kit by Illumina before being sequenced using Illumina's HiSeq platform. Bioinformatic analysis was performed using dropSeqPipe, a free and open source software for the rapid analysis and graphical expression of scRNA-Seq datasets.

Results

Encapsulating high viscosity protoplast buffers on Nadia

With real time visual feedback on droplet formation, we were able to confirm that stable droplet formation of WI buffer is maintained throughout the entire runtime (~15 mins)(Figure 3).

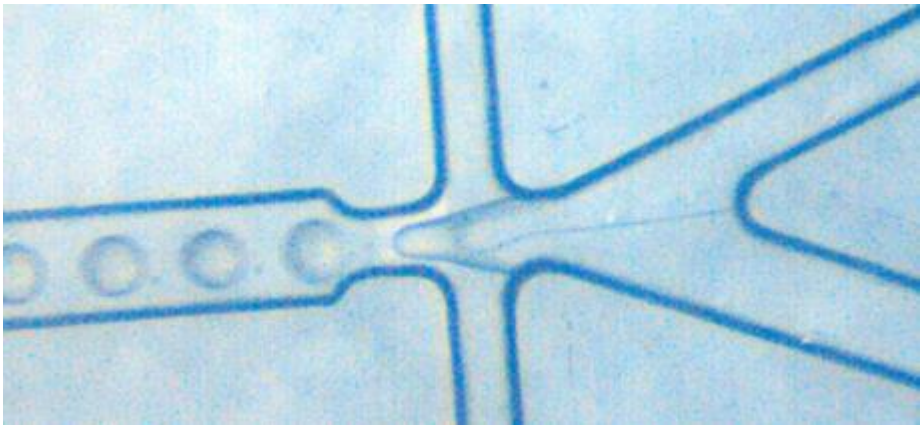

Figure 3 *Still image of microfluidic Nadia junction producing stable droplets from high viscosity WI buffer and lysis buffer with newly customised pressure profile (ppRNA-Seq.nadia).*

Droplet formation with W5 buffer required an alteration of the W5 buffer formula to omit $CaCl_2$, becoming W5-$CaCl_2$ buffer (Table 1). This swift optimisation process was made possible by the real-time visual feedback from the Nadia Innovate's high speed video camera and microscope. During encapsulation, precipitation was observed forming along the interface between the EDTA-rich lysis buffer and W5 buffer. Removal of $CaCl_2$ from the buffer solution allowed stable encapsulation without precipitation formation. The removal of $CaCl_2$ has no effect on the viability of leaf protoplasts suspended within, since the presence of $CaCl_2$ is purely to encourage transfection in plant cells [14,15].

As a result, two modified pressure profiles were created for encapsulating plant protoplasts on the Nadia platform in WI (ppRNAseq.nadia) and W5-$CaCl_2$ (ppRNAseq_W5-Ca2.nadia) buffers. Pressure values for the encapsulation steps

of these protocols are displayed in Table 2. These were ported onto the Nadia platform for use with multiple samples.

Table 2 *Pressure levels for dropletisation of protoplast-optimised buffers on the Nadia platform*

Protocol	Oil pressure (mbar)	Bead line Pressure (mbar)	Cell line pressure (mbar)
Standard scRNA-Seq on Nadia (1 x PBS)	450	140	133
ppRNAseq.nadia (WI Buffer)	400	146	188
ppRNAseq_W5-Ca2.nadia (W5-$CaCl_2$ buffer)	350	120	125

High throughput encapsulation of plant protoplasts on Nadia

Many single cell microfluidic workflows require the transferral of isolated plant cells into a low viscosity and low osmolyte PBS-based buffer prior to microfluidic capture alongside oligo beads. Here we performed encapsulation of plant leaf protoplasts with dummy oligo beads in high viscosity protoplast-friendly WI buffer to demonstrate that cell viability is maintained post-dropletisation on Nadia. The morphology of cells captured intact is indicative of leaf mesophyll protoplasts, whose size has previously made them a difficult target for other high-throughput microfluidic studies (Figure 4). Additionally, mesophyll protoplasts stored within this WI buffer do not divide [14], making this an ideal system for use in single cell genomic analysis.

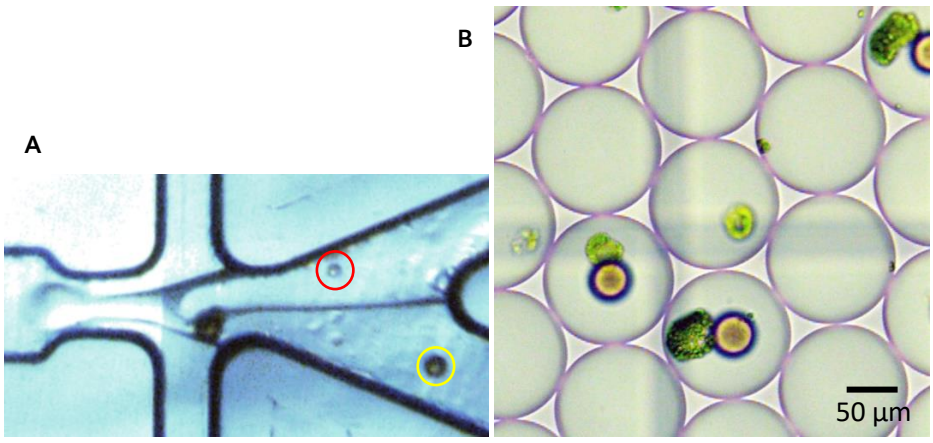

Figure 4: *(A) Still image of microfluidic junction generating stable droplets containing oligo coated beads (yellow circle) suspended in lysis buffer and A.thaliana mesophyll protoplasts (red circle) suspended in WI buffer. Imaged in real time using Nadia Innovate integrated high-speed digital microscope. (B) A sample of the resultant monodisperse droplets (~90 µm diameter) containing spherical hard oligo-dT capture beads and intact plant leaf protoplasts. Scale bar represents 50 µm.*

With these pressure profiles incorporated into the easily customisable protocols on the Nadia Innovate, protoplasts were able to be encapsulated in optimal

cellular conditions ready for single cell RNA-Seq or similar droplet-based protocols. In one step, the customised protocols can be uploaded to any Nadia or Nadia Innovate system, allowing users to encapsulate protoplasts in their choice of two buffers optimised for plant cell viability.

Arabidopsis thaliana leaf protoplast single cell RNA-Seq (ppRNA-Seq) on Nadia

ppRNA-Seq on Nadia produces high quality sequencing data and gene capture from plant leaf protoplasts

Singulated A.thaliana plant leaf protoplasts were encapsulated on the Nadia instrument using the newly created ppRNA-Seq on Nadia protocol alongside oligo-dT beads suspended in lysis buffer [13]. Libraries containing a presumptive 250 STAMPs (Single Transcriptomes attached to MicroParticles) were prepared for each plant buffer sample. This involved taking a subsample of the total number of bead-bound cDNA libraries from each sample. Unprocessed bead-bound libraries can be stored up to 4 weeks at 4°C ready for later analysis of the full ~6000 STAMPs per sample. Following library preparation and next generation sequencing, bioinformatic analysis using dropSeqPipe yielded important post-filtering metrics highlighted in Figure 5.

Dataset	Uniquely mapped reads (%)	Cells analysed	Read depth per cell	Mean genes per STAMP
Nadia ppRNA-Seq with WI Buffer	87.8%	250	88531	1908
Nadia ppRNA-Seq with W5-$CaCl_2$ buffer	69.1%	250	55504	1879

***Figure 5**: Summary table of single cell quality control metrics from ppRNA-Seq on Nadia of A.thaliana leaf protoplasts demonstrates general efficacy of ppRNA-Seq with both WI and W5-CaCl2 buffers.*

Analysis of these ppRNA-Seq datasets using dropSeqPipe allows the efficient mapping of reads to the A.thaliana transcriptome (Figure 5). Gene capture with ppRNA-Seq on Nadia (~1900 mean genes per STAMP) exceeds the documented gene capture for Drop-Seq within A.thaliana root tissue (~1579 genes per STAMP)[5]. Similarly, this level of gene capture approaches the ~2.5k genes per cell measured previously within developing leaf cotyledons of 5-day old Arabidopsis seedlings [16].

Since 250 STAMPs were easily identifiable within each library (Figure 5), generated from 5000 beads each, This indicates that ~6000 transcriptomes are attainable from each sample if all 120,000 beads are processed into cDNA. By extension, from a starting amount of 75,000 protoplasts per sample, the capture rate of ppRNA-Seq is roughly equivalent to that in standard scRNA-Seq on Nadia, at approximately 10%. The dataset derived from protoplasts in WI buffer was chosen for further analysis and standardisation into the ppRNA-Seq protocol.

ppRNA-Seq on Nadia captures the expected range of prospective cell types from *A.thaliana* leaf protoplasts

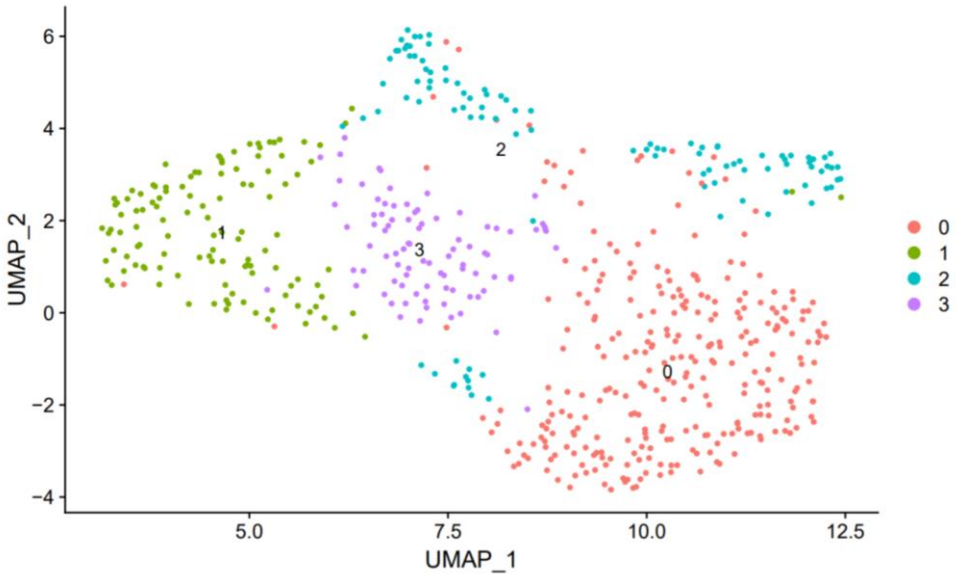

Figure 6: *Uniform Manifold Approximation and Projection (UMAP) visualisation of 250 STAMPs from single A.thaliana leaf cells encapsulated in WI buffer using ppRNA-Seq on Nadia reveals 4 distinct cell clusters. Two clusters (0 and 2) were associated with mesophyll cells and one cluster (1) with guard cells.*

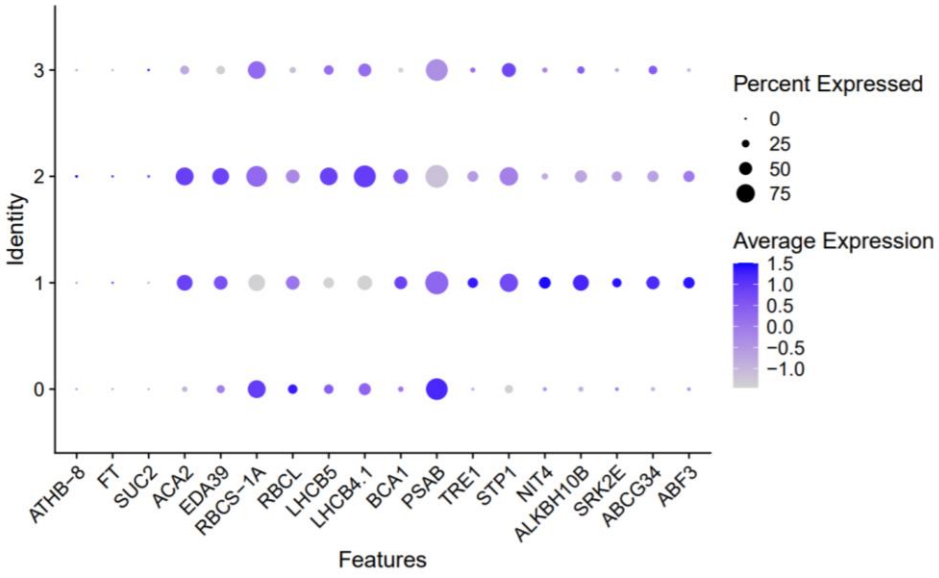

Figure 7: *Dot plot displaying the percentage of cells expressing selected genes (dot size) and average expression levels of genes based on transcript counts (colour intensity) for dataset used in Figure 6. Rows correspond to cell clusters by UMAP (Figure 6), presenting the expression of relevant cell type marker genes.*

Previous literature has identified abundant marker genes for mesophyll cells in adult *A.thaliana* leaf. Here (Figure 7) in each of our cell clusters, we show the expression levels of these mesophyll identity marker genes, RBCS-1A, LHCB5, LHCB4.1 [17], PSAB [16], BCA1 [18], ACA2 [19], RBCL [20] and EDA39 [21,22]. The enrichment of these chloroplastic and photosynthetic pathway genes in clusters 0 and 2 of our datasets (Figures 6 and 7) suggests that these groups represent

leaf mesophyll cells. The individual cellular and transcriptomic responses of both mesophyll and guard cells are vital targets for research concerning photosynthetic yield and desiccation avoidance [23] in the dynamic field of crop optimisation.

The division of clusters 0 and 2 into two distinct clusters could represent the subdivision of leaf mesophyll cells into spongy and palisade layers [24]. However, it is difficult to differentiate between these two cell types by single cell RNA-Seq data alone as few conclusive genetic markers for mesophyll cell sub-type are available [24].

The continued use of high throughput ppRNA-Seq on Nadia is likely to aid in the identification of genes specific to palisade or spongy mesophyll leaf cells. This would allow more effective delineation of these two similar sub-tissue cell types in future single cell research without relying on morphological examination [24].

Another major contingent of cells expected within leaf tissue isolates are the guard cells surrounding stomata. Though less abundant than mesophyll cells [25], their unique transcriptomic signature allows us to identify guard cells amongst the cell clusters in our dissociated *A.thaliana* leaf samples. STAMPs within cluster 1 (Figures 6 and 7) represent this guard cell population due to their enriched expression of SRK2E, ALKBH10B, ABF3, ABCG34, NIT4 [26], STP1 [27–29] and [25,26,29].

Vascular leaf cell types were not detected within our protoplast samples, as confirmed by the absence of leaf vasculature marker genes ATHB-8 [17], FT and SUC2 [30] in all cell type clusters (dot plot). This was expected from our samples (Figure 2), as the isolation technique used here omits the skeletal vasculature of *Arabidopsis* leaves [14]. Cell cluster 3 is so far unidentified, but could represent pavement cells, or more generally cells of epidermal origin (Figure 7). Epidermal cell identity would be continuous with their lower expression levels of photosynthetic activity genes when compared to clusters 0 and 2 [31].

Mesophyll-specific gene expression demonstrates that ppRNA-Seq on Nadia is effective for sequencing large plant leaf cells

A.thaliana leaf mesophyll cells can be as large as 60 µm in diameter once protoplasted, as evidenced by the large size of the cell isolates in our own leaf extracts (Figures 2 and 4B) and by confocal microscopy imaging of intact *A.thaliana* leaf tissues [1,8]. This large size has previously precluded plant leaf mesophyll tissues from analysis using high throughput single cell genomic analysis, due to their inability to fit reliably through conventional microfluidic junctions [9]. Considering that mesophyll contributes to approximately 80% of the RNA from whole leaf isolates [24], the inclusion of these cell types crucial for photosynthetic activity in high throughput single cell datasets is invaluable.

Clustered gene expression data indicate that 2 major clusters within our samples contain photosynthetically active cell types with mesophyll transcriptomic signatures (figures 6 and 7), showing that ppRNA-Seq on Nadia overcomes the issue of cell size to assay these large mesophyll cell types.

This is primarily due to the Nadia instrument's wide microfluidic junction, allowing cell types to be analysed which would be damaged or omitted by other droplet-based microfluidic platforms.

Conclusion

The unrivalled versatility of the Nadia Innovate allows users to swiftly modify parameters to suit buffers of widely varying viscosities. In addition, troubleshooting of problematic buffer components is made simple by the ability to view droplet formation and buffer mixing in real time. The new ppRNA-Seq on Nadia protocol can generate high quality single cell data from hundreds of single plant leaf protoplasts, specifically allowing single cell RNA-Seq datasets to be generated from large mesophyll leaf cells by microfluidic means. With these advances in accessible and high-throughput microfluidic technology, a new cohort of single cell genomics workflows for plant cells can be realised.

Acknowledgements

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Product Information

Nadia Innovate system

Description	Part number
Nadia Instrument	3200590
Nadia Innovate	3200595
Nadia Innovate Upgrade for Modular Systems	3200660

Nadia Innovate consumables

Description	Part number
Innovate Chips – 8 runs (8x1)	3200611
Innovate Chips – 40 Runs (40x1)	3200612
Innovate Cartridge – 8 runs (8x1)	3200597
Innovate Cartridges - 8 Runs (2x2 & 1x4)	3200598
Innovate Cartridge - 8 Runs (1x8)	3200599
Innovate Cartridges - 40 Runs (40x1)	3200613
Innovate Cartridges - 40 Runs (10x2 & 5x4)	3200614
Innovate Cartridges - 40 Runs (5x8)	3200615