APPLICATION NOTE

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# User-friendly automation of full-length single-cell RNA-sequencing with FLASH-seq

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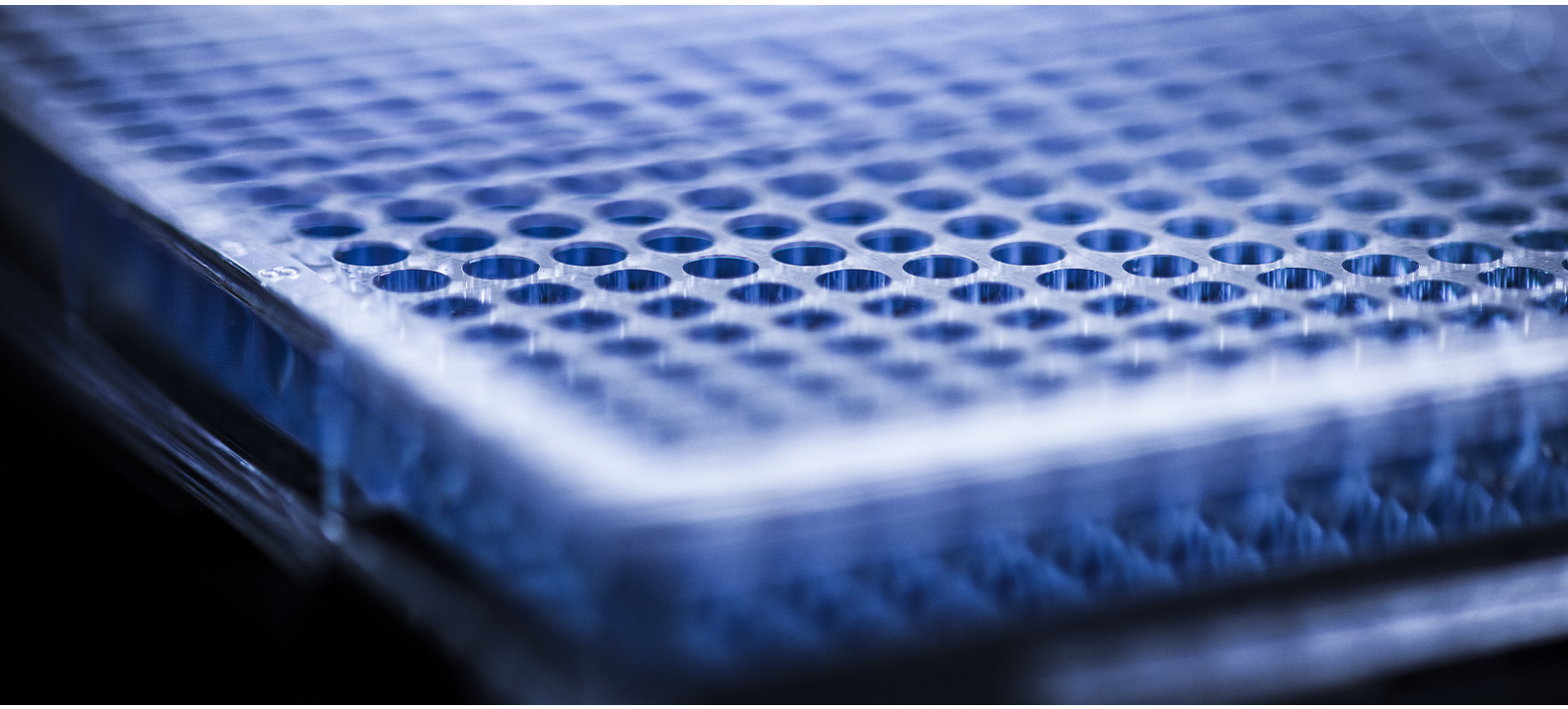


## Abstract

Full-length single-cell RNA-sequencing (scRNA-seq) is considered the gold standard in the field of transcriptomics, as it allows the characterization of gene isoforms, SNPs, mono- and diallelic gene expression as well as transcriptional start sites. Water-in-oil emulsion droplets do enable the sequencing of a much higher number of cells per experiment but bear at least one of the following limitations: lower number of genes detected per cell ("sensitivity"), reliance on commercial kits (locked systems), poor cell capture rate and, most importantly, retain only the terminal 3'-end information from each mRNA. Depending on the biological question, these methods can still be valuable in an unbiased analysis or when users have limited experience in single-cell genomics.

While nanoliter-scale reactions carried out in emulsion droplets allow the study of hundreds of thousands of cells in a single test tube, microliter-scale reactions and plate compartmentalization for full-length methods present serious drawbacks. Researchers from the Picelli Lab in collaboration with CYTENA have developed a fully

automated and user-friendly version of the recently published method called FLASH-seq in order to mitigate throughput and cost concerns. Using week-22 human retinal organoids (a complex in vitro model system), they have generated excellent results with minimal manual intervention. CYTENA's F.SIGHT™ OMICS and C.WASH™, as well as DISPENDIX's I.DOT™ have been seamlessly integrated in our workflow, shifting the focus from data generation to data analysis and interpretation.

# Introduction

scRNA-seq has transformed genomics in the last decade. Although limited in throughput compared to water-in-oil emulsion methods (i.e., 10X Genomics), plate-based methods still have their place in the lab thanks to their superior sensitivity and ability to provide full-length transcript information. Smart-seq2 remained for many years the gold standard among them.[1] Despite their usefulness, all plate-based methods share important drawbacks compared to water-in-oil emulsion methods: extensive hands-on time, low throughput and higher cost per cell.

To address most of these issues, we have developed FLASH-seq, a novel, full-length scRNA-seq method capable of generating sequencing-ready libraries in a single workday while providing superior data quality.[2] Here, we present a fully automated version of FLASH-seq where throughput is only dependent on reaction and incubation times, manual intervention is limited to the preparation of master mixes and almost all dispensing and cleanup steps are performed by the instruments. Protocol automation enhances reproducibility and data quality and decreases reagents' cost while increasing throughput and saving time. Notably, the reaction volumes indicated in this Application Note are meant as a suggestion only. For users familiar with protocol automation and miniaturization, further volume reduction is possible to cut reagent costs even more (see Fig. S2i).[2]

# Material and methods

## Single-cell isolation

Retinal organoids were generated from the induced pluripotent stem cell (iPSC) line 01F49i-N-B7.[3] At week 22, retinal organoids were selected based on the presence of outer segments and characteristic retinal layers. Retinal parts of organoids were dissected and maintained in 3:1 medium supplemented with N-2 (Gibco) at 37°C until dissociation. Dissected organoids were pooled together and washed once with 1 ml Ringer solution without calcium. The Neural Tissue Dissociation Kit P (Miltenyi Biotec) was used to dissociate organoids into single cells, following the manufacturer's instructions. The resulting cell suspension was spun for 5 minutes at 300 x *g* in a pre-refrigerated centrifuge (Eppendorf), the supernatant was removed and the pellet was washed once with 1 x PBS. Cells were resuspended in 1 x PBS before being strained using first a 70µm and then a 40µm filter. To label dying cells, an aliquot was stained with a 1:500 dilution of propidium iodide in 1 x PBS.

Single cells were isolated using the F.SIGHT OMICS single-cell dispenser (described in detail below). All plates contained 2 negative controls (in wells A1, A12) and 2 positive controls (in wells P12, P24). After sorting, plates were sealed with aluminum foil seals (VWR) and immediately placed in a -80°C freezer until ready to be further processed.

# Lysis buffer preparation and dispensing

Lysis buffer was dispensed in 384-well plates using S.100 source plates on the I.DOT non-contact liquid handler and stored at -20° until sorting day. The lysis buffer used in the experiment had the following composition: 0.02 µl Triton X-100 (10% v/v, Sigma-Aldrich), 0.24 µl dNTP mix (25 mM each, Roth), 0.018 µl FS-dT$_{30}$VN (5´Bio-AAGCAGTGGTATCAACGCAGAGTACT$_{30}$VN-3´; Bio = biotin; 100 µM, IDT), 0.03 µl RNase inhibitor (40 U/µl, Takara), 0.012 µl dithiothreitol (100 mM, ThermoFisher Scientific), 0.2 µl betaine (5 M, Sigma-Aldrich), 0.09 µl dCTP (100 mM, ThermoFisher Scientific), 0.092 µl FS-TSO (5′ Bio-AAGCAGTGGTATCAACGCAGAGTACrGrGrG-3′, Bio = biotin; 100 µM, IDT) and water to 1 µl final volume. Of note, dCTP and FS-TSO can be included in the RT-PCR mix (instead of the lysis mix) without negatively affecting the overall results.

# cDNA preparation, purification, QC, and quantification

Plates were removed from the -80°C storage, transferred to a pre-heated Mastercycler thermocycler (Eppendorf), incubated for 3 minutes at 72°C and then placed on a metal block kept in an ice bucket. Four microliters of RT-PCR mix were added with the I.DOT using the S.100 source plate. The RT-PCR mix had the following composition: 0.238 µl dithiothreitol (100 mM), 0.8 µl betaine (5 M), 0.046 µl magnesium chloride (1 M, Ambion), 0.096 µl RNase inhibitor (40 U/µl), 0.05 µl Superscript IV (200 U/µl, ThermoFisher Scientific), 2.5 µl KAPA HiFi Hot-Start ReadyMix (2 x, Roche) and nuclease-free water to 4 µl final volume.

Plates were sealed, briefly vortexed (2000 rpm, 10 sec) and centrifuged (800 x g, 1 min) before being placed in the thermocycler for the RT-PCR reaction (~3h30): 60 minutes at 50°C and 3 minutes at 98°C, then 21 cycles of 20 seconds at 98°C, 20 seconds at 67°C and 6 minutes at 72°C, held at RT. Plates were cleaned up using SeraMag SpeadBeads (GE Healthcare) containing 18% w/v polyethylene glycol MW = 8000 (Sigma-Aldrich). Four microliters of beads (0.8:1 ratio of beads:cDNA) were dispensed by using a S.200 source plate with the I.DOT, sealed, quickly vortexed and then placed on a SmartBlock (Eppendorf) carrying a 384-well adaptor. Plates were incubated for 5 minutes with agitation (2000 rpm) at RT, spun down and then directly placed on the C.WASH magnetic carrier for 5 minutes to collect the beads. Then, the C.WASH noncontact centrifugal plate washer was used with a custom program for liquid removal and elution of cDNA. The plate was centrifuged for 10 seconds at 20 x g to discard the supernatant before 15 µl of nuclease-free water were dispensed into each well. After sealing, plates were briefly vortexed and then placed on the SmartBlock for 2 minutes, with agitation (2000 rpm, at RT). A Fluent liquid handling robot (Tecan) was used to transfer the eluted cDNA into a new 384-well plate.

Sample cDNA concentration was measured with the Quant-iT PicoGreen dsDNA Assay kit by transferring 1 µl cDNA into 80 µl of a 1:400 dilution PicoGreen using the Fluent robot, before measuring the fluorescence with the Hidex Sense instrument (Hidex). To assess cDNA size distribution, eleven samples were randomly selected from each plate, loaded onto a High Sensitivity DNA chip and run on a 2100 Bioanalyzer System (Agilent). High quality cDNA is characterized by the absence of short fragmented (<500 bp) and by a narrow size distribution centered around 2 kb.

## NGS library preparation

Samples were normalized to a final cDNA concentration of 150 pg/µl with the I.DOT before transferring 1 µl of purified and normalized cDNA to a new plate for library preparation. With the I.DOT 3 µl of tagmentation mix, 0.8 µl 100% dimethylformamide (Sigma-Aldrich), 0.8 µl 5 x TAPS buffer (50 mM TAPS-NaOH pH 7.3 at 25°C, 25 mM $MgCl^2$), 0.05 µl Tn5 transposase (~2 µM) and nuclease-free water to volume were added to each well. Plates were incubated for 8 minutes at 55°C before adding 1 µl 0.2% SDS with the I.DOT. After vortexing and spinning down, samples were left at room temperature for 5 minutes to allow the SDS to properly release the Tn5 from the cDNA. The enrichment PCR mix was then dispensed into each well, again with the I.DOT: 0.2 µl KAPA HiFi DNA Polymerase (1 U/µl), 0.3 µl dNTP mix (10 mM), 2 µl 5X KAPA HiFi Buffer (all part of the KAPA HiFi PCR kit, Roche) and water to 3 µl final volume. To uniquely index each cell, 2 µl pre-mixed custom N7xx and S5xx index adaptors (5 µM each) were added by using the Fluent robot before carrying out the PCR reaction: 72°C for 3 minutes, 95°C for 30 seconds, then 14 cycles of 95°C for 10 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by 72°C for 5 minutes and a 4°C hold. Afterwards, all cells were pooled together in a 1.5-ml tube before performing the cleanup with 0.8X of SeraMag beads. Purified libraries were resuspended in nuclease-free water and kept at -20°C until ready for sequencing.

A large set of Illumina-compatible index adaptors (64 i7xx and 48 i5xx, IDT) were resuspended in low-EDTA TE buffer to a final concentration of 10 µM, transferred in rows (i7xx) or columns (i5xx) of 2 separate S.100 source plates and multiple working dilution plates were prepared in a single round using the I.DOT, by mixing equal volume of the 2 sets in all possible combinations.

## Sequencing and data processing

Samples were sequenced on a NovaSeq 6000 instrument using a 100-cycle flow cell and single-end (SE) read mode.

After read mapping, low-quality cells were defined as harboring either less than 100,000 uniquely mapped reads, less than 70% uniquely mapped reads, greater than 3,000 expressed genes or greater than 20% multi-mapped reads. Negative and positive controls were excluded as well. Data analysis was performed as previously described,[1] except for the read depth normalization, where Seurat *SCTransform* was replaced by the default log-normalization implemented in *ScaleData*.

# Results and discussion

## User-friendly and fully automated FLASH-seq protocol

Full-length scRNA-seq protocols are often laborious due to the large number of pipetting steps required, especially when large numbers of cells need to be analyzed. While the recent chemistry improvements introduced with FLASH-seq reduce these steps to a minimum, processing a full 384-well plate remains intimidating for most laboratories lacking automation. Moreover, sorting individual cells into a 384-well plate can be challenging even for many FACS experts. To tackle these issues, we set up a fully

automated FLASH-seq protocol based on the latest line of instruments developed by CYTENA and DISPENDIX. We combined the F.SIGHT OMICS, C.WASH and I.DOT into a user-friendly pipeline that can be operated without any prior knowledge in liquid-handling robot programming or FACS expertise.

We initially used DISPENDIX's I.DOT noncontact liquid handler to dispense 1 µl of mild hypotonic lysis buffer in 384-well plates using the platform's S.100 source plates and stored them at -20° until sorting day. The I.DOT can easily dispense such volumes while manual pipetting would be very cumbersome, especially when working with solutions containing detergents. The wide dynamic range of the I.DOT (8 nl to 80 µl, S.100 plates) guarantees full flexibility in terms of reaction volumes, thus accommodating any possible variation of the standard workflow, both in terms of volume as well as input material (1-1,000 cells).

On the day of the experiment, dissociated retinal organoid cells were deposited into the lysis buffer using the F.SIGHT OMICS. This low-pressure benchtop single-cell dispenser minimizes the stress associated with sorting, giving researchers the opportunity to conduct experiments with very fragile cells that would not have been possible with a FACS instrument. Eighty microliters of cells resuspended in PBS (500,000 cells/ml) were loaded on a disposable one-way cartridge featuring a 40 µm nozzle. Cells were prevented from settling through agitation. System booting, droplet QC and sorting layout setup took fewer than 20 minutes, a gain of over 1 hour compared to a standard FACS experiment, which usually requires manual adjustments of sorting parameters such as fluorescence intensity, forward and sideward scatter, and droplet delay. Moreover, the device is equipped with a system for automatic offset correction (AOC) to bypass the need for manual alignment of droplets to the target positions of the substrate, which allows targeted, reproducible dispensing of individual cells to the bottom of PCR plates. This helps to further reduce hands-on time and allows for cell lysis in minute amounts of lysis buffer (0.5 µl to 1 µl), which is the prerequisite for flexible downscaling of reaction volumes in plate-based workflows. Overall, operating the device was straightforward and required only minimal training (<30 minutes).

Due to the limited amount of starting material and leveraging the fact that our cell suspension contained a very high proportion of live cells (~95%) with relatively little debris, we opted for sorting cells into plates without using any fluorescent marker, relying exclusively on size (8-16 µm) and roundness (>0.6) instead. Thanks to this high cell viability, it took roughly 8 minutes to sort single cells in all the wells of a 384-well plate. Once finished, shutting down the F.SIGHT OMICS did not require extensive washes and was completed in under 5 minutes, in contrast to the significantly longer wash cycle required for a thorough cleaning of both sample line and cuvette flow cell on a regular FACS instrument.

After sorting and RNA heat denaturation, the RT-PCR master mix was dispensed using the I.DOT (S.100 source plate) and the reaction was carried out in a thermocycler. The amplified cDNA was purified with magnetic beads using both the I.DOT and the C.WASH. Using these two devices instead of a more complex and expensive liquid handling robot saves one to two 384 tip boxes for each plate (~$0.4-0.8 / cell), depending on the workflow. In the final elution step, the clean cDNA was transferred into a new plate using a Fluent liquid handling robot. The cDNA concentration was measured with the Quant-IT PicoGreen dsDNA Assay kit and then diluted in preparation for sequencing. We wrote an R-script that calculated the required amount of water to dilute 1 µl of purified cDNA to a final concentration of 150 pg/µl (based on

the PicoGreen measurements) and generated an I.DOT-compatible CSV file. This CSV file was used to dispense different volumes of water into a new plate containing 1 µl cDNA/well that had been previously dispensed with the Fluent robot.

This normalized cDNA plate was used to generate the final sequencing library in a two-step process. First, 1 µl of diluted cDNA was mixed with the tagmentation mix dispensed with the I.DOT (S.100 source plate). The tagmentation reaction was carried out using a homemade Tn5 transposase to reduce costs and ensure full protocol flexibility. After incubating and quenching the reaction on ice, 0.2% SDS was added with the I.DOT to remove the Tn5 from the tagmented cDNA. Second, the enrichment PCR mix was added with the I.DOT (S.100 source plate), while index primers were added separately using the Fluent robot. Although researchers' needs vary among labs, we found it very convenient to generate index plates with pre-mixed and ready-to-use i5 and i7 adaptors at approximately 5 µM in a 384-well format that can be stored in the freezer until needed. Multiple freezing and thawing rounds of the adaptor plates did not have a negative impact on the overall performance. The cross contamination risk is lower compared to manual protocols if the instruments are kept clean and undergo the periodical maintenance. The final library products were pooled into a single tube and purified by bead cleanup, as described above.

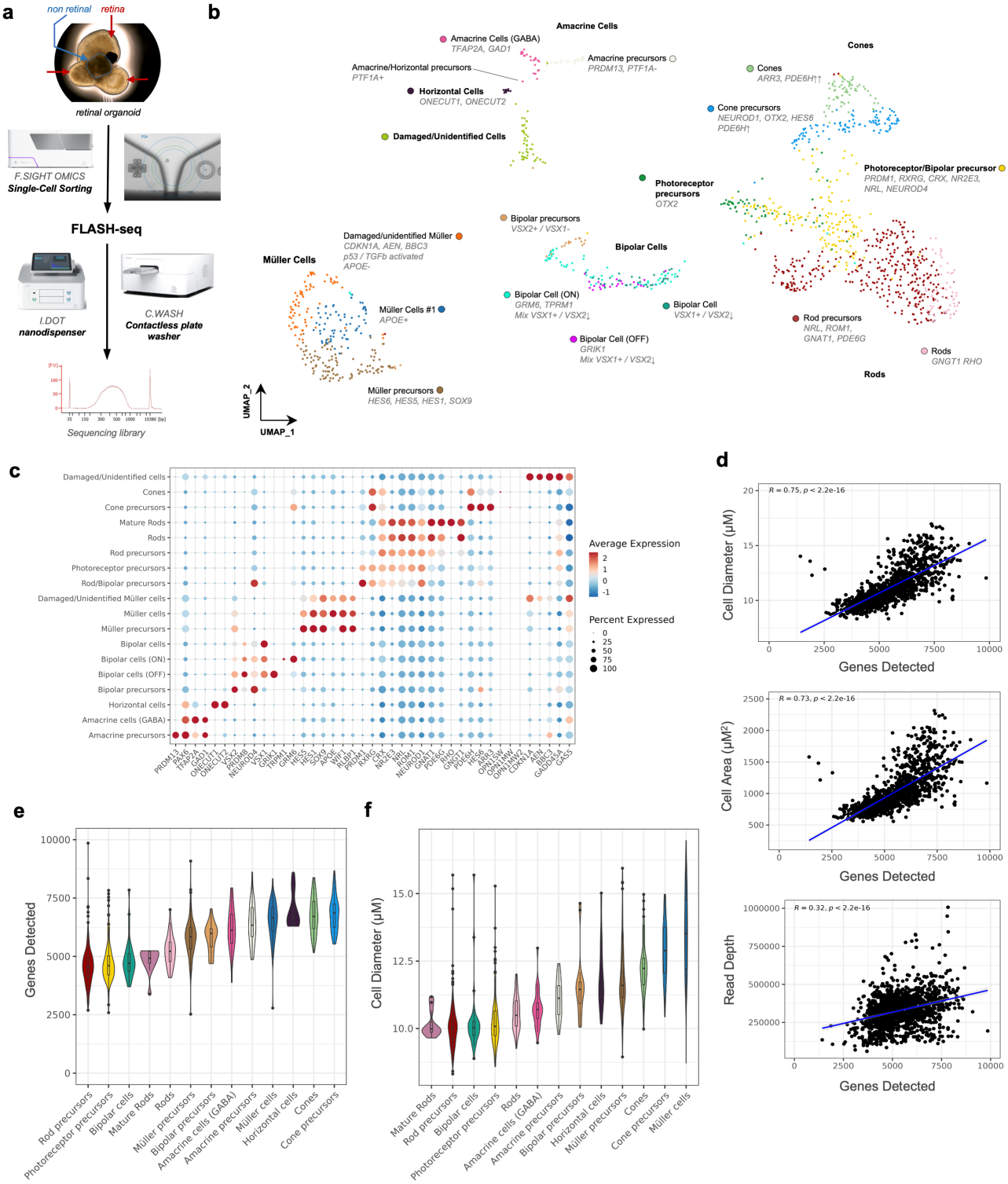
# Week-22 retinal organoids

To evaluate the impact of these new instruments on the FLASH-seq protocol, we used week-22 retinal organoids for our benchmarking. Retinal organoids are light-responsive and recapitulate the cell type diversity / functions of the retina.[3] At this developmental stage and after selecting the retinal parts (Fig. 1a), six major cell types are expected: amacrine cells, bipolar cells, horizontal cells, Müller cells, rod cells and cone cells. We processed four full 384-well plates (~1536 cells) according to the protocol described above. On average, 91.4±4% of the sorted wells had a cDNA value greater than 0.5 ng/µl, showing a high sorting efficacy similar to our previous FACS experience.

Cells were sequenced at an average depth of 613,799±190,679 reads (median±SD). After filtering out low-quality cells, we recovered 1,300 cells (84.6%). This value is similar to the success rate observed for week-18 retinal organoids cells isolated by FACS (1,281, 83.4%).[2]

Manual cell type annotation revealed the six expected cell types (Fig. 1b-c). All subtypes still showed signs of ongoing differentiation. As previously described,[3] ganglion cells (RBPMS+ and/or SNCG+) were undetectable at this developmental stage. In addition, no astrocyte (GFAP+) or glycinergic amacrine (SLC6A9+) cells could reliably be identified. A subset of cells harbored cell cycle arrest markers and overexpression of genes associated with the intrinsic apoptotic signaling pathway (GO:0097193, e.g., BBC3, BCL2, TNF, CDKN1A, etc.) and were labeled as "damaged/unidentified".

We recovered 4,553±819 expressed genes (rod progenitors, [median±SD]) to 6,868±734 (cone progenitors) thanks to the high sensitivity of FLASH-seq (Fig. 1e). The number of detected isoforms and gene diversity was also in line with previous results in week-18 retinal organoids.[2]




## Figure 1.

**a.** *Retinal organoid and schematic overview of the protocol.*
**b.** *UMAP of the different cell populations with key cell type markers highlighted.*
**c.** *Dotplot of cell type markers.*
**d.** *Relationship between the number of genes detected and the cell diameter (µm), cell surface area (µm²) and read depth. Pearson correlation coefficient and associated P-value displayed in the top left corner.*
**e.** *Genes detected per selected cell types.*
**f.** *Cell diameter (µm) per selected cell type.*

In addition to simplifying cell sorting, the F.SIGHT OMICS enabled the recording of some additional parameters that could be used in the downstream analysis. First, physical parameters such as diameter, area or roundness are recorded for each single-cell event. We observed a strong correlation between the number of genes detected and cell size (Pearson coefficient > 0.73, P-value < 2e-16, Fig. 1d) but not with its roundness (p-value = 0.3). Müller cells and cones are among the largest cells, confirming visual and immunohistochemistry reports previously made in our laboratory. This correlation was far more important than with the sequencing depth (Pearson coefficient = 0.32, P-value < 2e-16, Fig. 1d). Interestingly, each cell type displayed different median cell sizes (Fig. 1d), such that cell size could likely be used for enriching specific cell populations or as a cell type prediction parameter.

Second, the F.SIGHT OMICS software stores five consecutive images of each sorting event, which allows researchers to visually inspect cells for the presence of smaller debris or unusual structures (Fig. 2). This feature is very relevant in the case of retinal organoids where single cells are obtained by enzymatic dissociation, which tends to sever photoreceptor inner/outer segments. These cell fragments can then "stick" to other cells present in the suspensions and give origin to clustering artifacts due to the merging of two cell types. Although not flawless, the 2D imaging potentially allows users to re-evaluate each sorting event and manually inspect cells from suspicious clusters.

Third, even though this feature was not used for this experiment, fluorescence values are attributed to each cell, enabling sorting of specific subpopulations to fulfill predetermined thresholds and cutoffs.

# Conclusions

The C.WASH, I.DOT and F.SIGHT OMICS instruments are versatile tools that can be used to automate most steps of the FLASH-seq workflow and greatly simplify plate handling. They are especially relevant for small- and medium-sized labs, as the upfront investment required is smaller compared to FACS or other liquid handling robots, thus lowering the barrier of entry for automation. In addition, the contactless bead cleanup on the C.WASH decreases the dependance on plastic tips, which have become a rare commodity nowadays and comes with an extra cost of $0.4-0.8/cell.

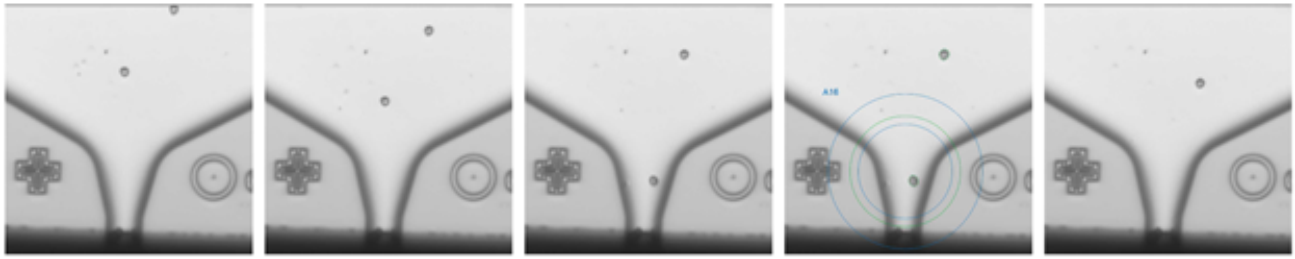
All instruments displayed here are easy to use, need minimal training and maintenance from researchers and do not require users to learn any programming language. Despite the minimalistic software, long and/or complex programs can be devised as highlighted by the cDNA dilution protocol or the generation of pre-mixed, ready-to-use index adaptor plates, a task that would have been more error prone, time consuming and expensive with a standard liquid handling robot and altogether unfeasible by hand.

However, these instruments cannot alone fully replace a liquid handling robot, which is still needed for some plate-to-plate transfer steps. Manual pipetting with multi-channel pipettes is manageable for half-full 384-well plates but is not recommended for full plates. One alternative for reducing the burden of manual pipetting when no liquid handling robot is available is to use the FLASH-seq low-amplification protocol (FS-LA).[2] In addition to shortening the workflow by roughly 2-3 hours, FS-LA skips the intermediate cleanup step, sample QC and normalization.

This leaves only two transfer steps instead of four: transferring the amplified and unpurified cDNA to a new plate for the tagmentation reaction and adding index primers before the final enrichment PCR reaction. We decided against FS-LA for this experiment, as we wanted to run the sample QC and normalization steps and observe the impact (or lack thereof) of these new instruments on the reaction.

In conclusion, the three instruments presented in this report can be seamlessly integrated to allow significant workflow miniaturization and subsequent amortizing without the typical financial investment required for their purchase, making automation more accessible to smaller labs.

**a**



**b**

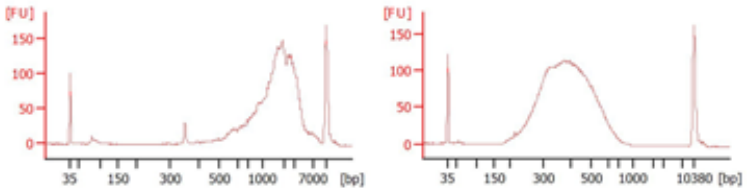



**Figure 2.**

**a.** *Time lapse of single-cell deposition. Note how a single cell is approaching the interrogation point proximal to the nozzle (images 1-3), it is correctly identified (4) and then disappears once it has been correctly deposited on the plate (5).*
**b.** *Bioanalyzer plot showing amplified cDNA derived from a single cell after RT-PCR (left) and the final indexed and pooled library (right).*

# References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