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Protocol

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Single-cell RNA-seq of maize meiocytes and pollen grains

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Abstract

RNA-sequencing (RNA-seq) provides invaluable knowledge on developmental pathways and the effects of mutant phenotypes. Plant reproductive cells have traditionally been difficult to isolate for genomics because they are rare and often deeply embedded within somatic tissues. Here, we present a protocol to isolate single maize meiocytes and pollen grains for RNA-seq. We discuss how to identify and isolate each sample type under a microscope, prepare RNA-seq libraries and analyze the data. This technique has several advantages over alternative methods, combining the ability to target specific rare cell types while resolving cell-to-cell heterogeneity with single-cell RNA-seq. The technique is compatible with minute amounts of starting material (e.g., a single anther), making it possible to collect dense time courses. Furthermore, developmentally synchronized anthers are saved for microscopy, allowing staging to be performed in parallel with expression analysis. Up to 200 cells can be collected in 4–5 h by someone proficient in tissue dissection, and library preparation can be completed in 2 d by researchers experienced in molecular biology and genomics. This protocol will facilitate research on plant reproduction, providing insights critical to plant breeding, genetics and agriculture.

Key points

- In this protocol, maize meiocytes and pollen grains are microscopically identified and isolated for single-cell RNA-seq by using a modified CEL-Seq2 workflow. Developmental staging of anthers is also performed in parallel with expression analysis.
- Rare reproductive cells are specifically targeted, and larger cell sizes and sample numbers are accommodated than in droplet-based single-cell RNA-seq methods. This protocol is compatible with minute amounts of material and resolves heterogeneity by focusing on single cells and pollen grains.

Key references

Nelms, B. & Walbot, V. *Science* **364**, 52–56 (2019): https://doi.org/10.1126/science.aav6428

Nelms, B. & Walbot, V. *Science* **375**, 424–429 (2022): https://doi.org/10.1126/science.abl7392

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Introduction

Plant reproductive research has led to transformative advances in plant breeding and genetics[1,2], which is increasingly important in the face of climate change and population growth. Single-cell RNA-seq (scRNA-seq) provides an unprecedented opportunity to study the development and function of reproductive cells such as meiocytes. Isolating reproductive cells for genomics has traditionally been challenging because the cells are often rare, large and deeply embedded within somatic tissues. This protocol describes how to isolate single maize meiocytes, pollen precursors and mature pollen grains for RNA-seq[3,4]. Pollen development is a complex, multistage process[4]. Our protocol is compatible with staged material from before meiosis through pollen shed (a 28-d long process in maize[4]). Slightly different techniques are used for tissue dissociation during meiosis compared with after, and the protocol explains both. In the last week of pollen development, pollen precursors become multicellular. Individual pollen cell types (the vegetative cell, generative cell and sperm cells) are not separated by this procedure, and so samples collected late in pollen development are single gametophytes (e.g., individual tricellular pollen grains) rather than single cells. Beyond maize pollen development, this protocol could be adapted to other species and cell types (see Applications) and is useful for many situations in which the cell type of interest is rare or material is limiting.

Overview of the protocol

The scRNA-seq protocol can be divided into four stages (Fig. 1). First, in Stage 1, floral tissue is dissociated to release reproductive cells. For meiotic cells, the anthers are fixed, and then meiocytes are released from the fixed tissue by simple mechanical disruption (Step 5A). For pollen grains and post-meiotic pollen precursors, fresh anthers are cut in half, and the loosely associated pollen readily releases into the liquid (Step 5B). During tissue collection, matched anthers from the same floret are fixed and saved for later microscopic staging. In Stage 2 (Steps 6–16), meiotic cells and pollen grains are identified under a microscope and manually isolated by using a blunt-end syringe needle. Single cells and pollen grains are then transferred to each cap of an eight-tube PCR strip and flash-frozen in liquid nitrogen for storage. This stage requires no specialized equipment other than a low-magnification inverted microscope. In Stage 3 (Steps 22–57), RNA-seq libraries are prepared with a modified version of CEL-seq2 (ref. 5). These are transcript-counting libraries that use 3′ tagging and unique molecular identifiers[6] (UMIs) to estimate transcript abundance (e.g., the number of mRNA molecules detected). Finally, in Stage 4 (Steps 58–63), the libraries are sequenced, and the data are mapped to the transcriptome and quantified.

Development of the protocol

This protocol was initially established to study the transition into meiosis[3], as progenitor archesporial cells complete their mitotic divisions and become pollen mother cells. We chose scRNA-seq to avoid pooling cells that may or may not be at the same stage based on gene expression, because this was not known a priori. This turned out to be important, because we found the most significant change in gene expression occurred within the classic cytological stage of leptotene; if we had pooled samples by the known staging, this transition would have been missed. We next applied the method to pollen development[4] and established the timing of haploid pollen genome activation. This second study used fixed meiotic cells rather than protoplasts. This was an important methodological advance because anther protoplasting was inconsistent and inefficient. After fixation, meiotic cells could be released with simple mechanical force (Supplementary Video 1), and high-quality scRNA-seq libraries could be obtained from these fixed cells[4].

Since our last publication[4], we have updated the primers used in our protocol so that the Illumina libraries now include Unique Dual Indexes (UDIs)[7]. The current generation of Illumina instruments (any instrument using patterned flow cells, including the HiSeq 4000, Novaseq, NextSeq and MiSeq) suffers from an index-hopping artefact in which multiplexed sample barcodes can be misassigned during sequencing[8] (also see the supplementary discussion

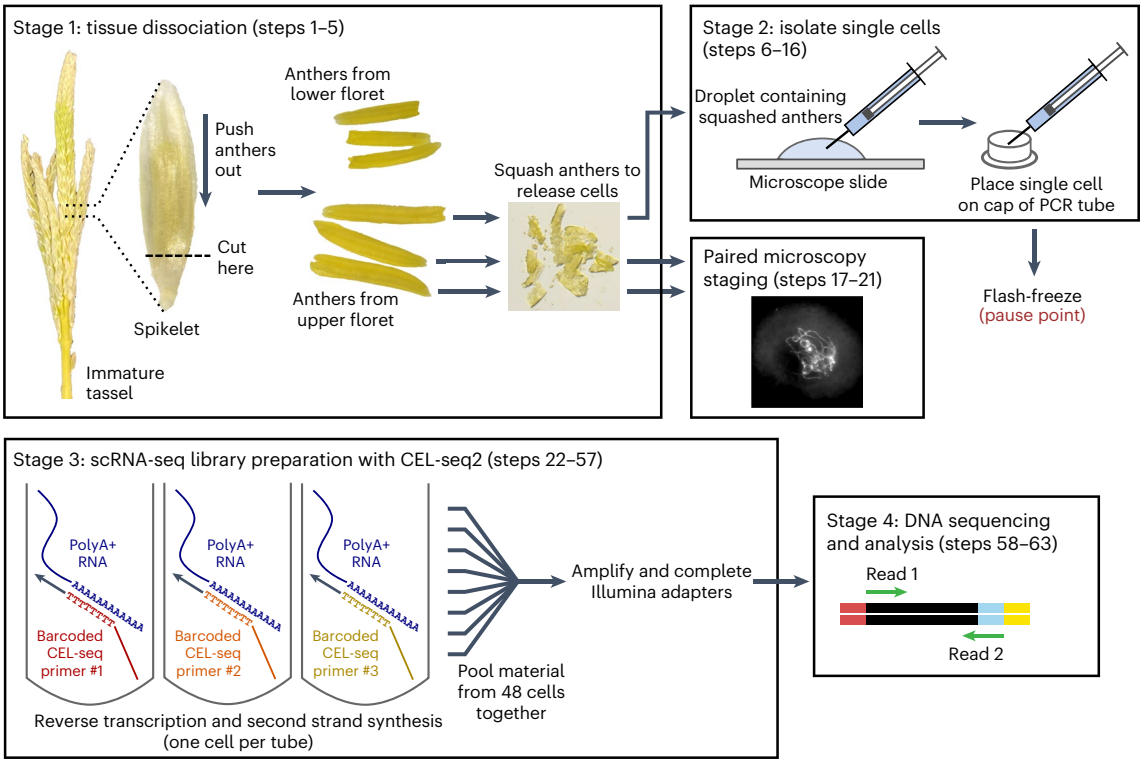

Fig. 1 | Overview of the protocol. Stage 1, tissue dissociation: anthers can be obtained by cutting the bottom of the spikelet and pushing them out carefully. Each spikelet contains two florets with three anthers each. Anthers in the upper floret are ~2 d ahead of the anthers in the lower floret in development. An anther can be collected from either floret for cell isolation, but it is important that paired anthers for microscopy staging are also collected from the same floret. Stage 2, cell isolation and microscopic staging: cells are isolated under a microscope by using a syringe needle and placed individually into the caps of PCR tubes. Paired material is saved and staged by microscopy, providing independent staging information that can be directly compared with scRNA-seq data. Stage 3, library preparation: single-cell RNA-seq libraries are prepared with a modified version of CEL-seq2; this protocol adds cell-specific DNA barcodes during reverse transcription, allowing material to later be pooled so that 48 cells can be processed together. Supplementary Fig. 2a shows an example reverse transcription primer with the barcodes annotated. Stage 4, DNA sequencing and analysis: finally libraries are sequenced, demultiplexed and mapped to the reference genome. Supplementary Fig. 2b shows the structure of a completed CEL-seq library with the location of the unique molecular identifiers, cell barcodes and transcript sequence annotated.

in ref. 4). Because of the high amount of multiplexing in single-cell libraries, this artefact can be substantial. The original CEL-seq2 protocol produced libraries with a single Illumina index (i5); our revised protocol allows for dual indexes (both i5 and i7). By using unique combinations of Illumina indexes during multiplexing ('UDIs'; e.g., each i5 index is always paired with a specific i7 index), sample labels that are misassigned because of index-hopping can be excluded[7].

Applications

Single-cell and single–pollen grain RNA-seq provides incredible resolution on gene expression and cell stages during plant reproduction. We have used this protocol to establish the timing of gene expression changes during meiosis[3] and pollen development[4] and to discover that the haploid gametophyte genome is not widely activated until 10 d after meiosis[4]. A key advantage of our approach is that tissue staging information can be collected in parallel with single cells (Fig. 1). Meiotic progression occurs synchronously along an anther in many plant species[4,9–11], including common research models such as rice[10] and *Arabidopsis*[11], and so our approach for meiotic staging could be directly adapted to other species.

This technique could also be applied to other cell types that are identifiable under a microscope. We see particular value for applications in plant reproduction, because many reproductive cells are very large and/or rare within their tissues. Cells can be identified by fluorescent markers in addition to morphology (e.g., *Arabidopsis* egg cells in Fig. 2), yielding

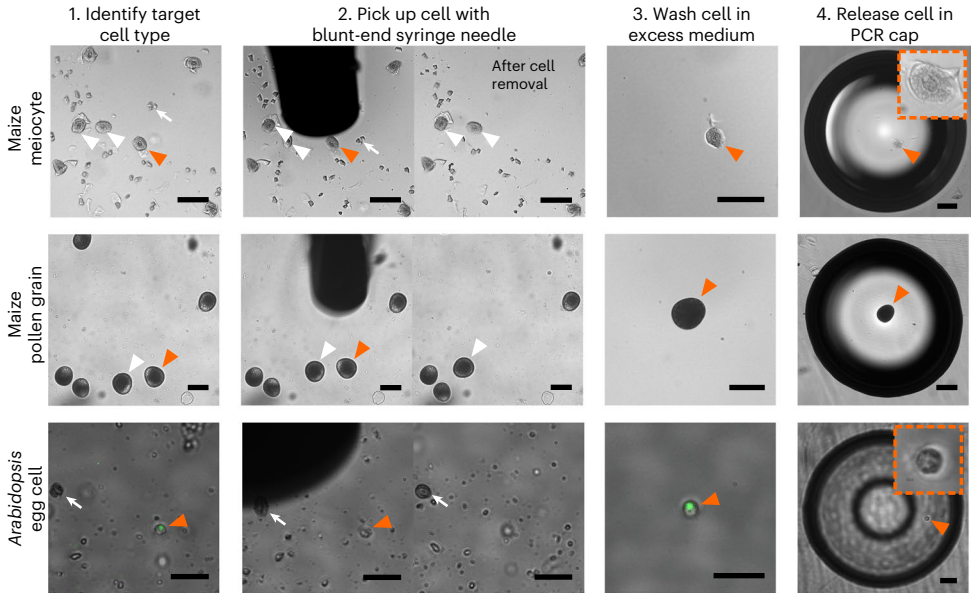

Fig. 2 | Single-cell isolation. An example of the isolation procedure for a maize meiotic cell is shown in the top row. Meiotic cells (arrowheads) can be identified by their large size relative to somatic cells and debris. One meiotic cell is picked up (orange arrowhead) along with tapetal cells (white arrow). The target meiotic cell is then isolated and washed before being put in the cap of a PCR tube. The second row shows a similar example for maize pollen grains. The third row shows how this technique can be adapted to other cell types and that it is compatible with identifying cells by using fluorescence. Here, an *Arabidopsis* egg cell is identified on the basis of nuclear localized YFP expressed under the DD45 promoter[25]. The first and fourth columns of the third row show merged images with both brightfield and fluorescence. Arrowheads, target cells. Orange arrowheads, target cell picked up. White arrows, examples of unwanted cell types. The images in the top and middle panels were obtained by using an inverted microscope (Olympus) with a ×10 objective and transillumination; the images in the bottom panels were obtained by using an inverted microscope (Zeiss) with a ×20 objective and transillumination (brightfield) or epiflourescence (GFP filter set; wide spectrum mercury light source). Scale bars: rows 1 and 2, 100 µm; row 3, 50 µm.

flexibility in the method used to select the cell type(s) of interest. Both protoplasts[3] and fixed cells that are mechanically isolated[4] can produce quality libraries with this method. In our experience with meiotic cells, fixed cells passed quality control slightly more often than protoplasts (90.8% of cells compared with 78.6%), but both had high quality and led to equivalent biological conclusions[3,4]; when establishing this technique with other cell types or species, we recommend using whichever tissue dissociation method is most robust in their system. This protocol can also be applied to small multicellular structures, which in addition to pollen, might include other applications in plant biology such as somatic embryos. Finally, other genomic methods could be applied to cells isolated with this protocol, including single-cell DNA-seq[12] or assay for transposase-accessible chromatin with sequencing[13].

Comparison with other scRNA-seq methods

Droplet-based methods for scRNA-seq, such as the 10× Genomics' Chromium chip, can collect thousands of cells in a very short timeframe[14]. By comparison, this protocol isolates fewer cells (hundreds rather than thousands) but can specifically target rare reproductive cells, accommodate larger cell sizes and collect cells from a greater number of distinct samples (e.g., multiple different stages and mutants):

- *Cell number*. Droplet methods have an advantage when large cell numbers are needed (>1,000), which can be important when distinguishing multiple different cell types of an entire organ. This protocol can accommodate hundreds of cells, which is often sufficient when focusing on a single cell type or incorporating independent staging information (e.g., microscopy).
- *Sample number*. Although droplet scRNA-seq can collect more cells, this protocol makes it easier to isolate cells from many distinct samples (e.g., tissues at different developmental

stages or genotypes). For instance, we previously collected cells from 104 staged anthers to provide a dense time course of expression throughout pollen development[4]. By comparison, droplet scRNA-seq requires a substantial material investment, and it is typical to assay only 2–10 samples per study.

- *Cell targeting*. This protocol makes it possible to isolate specific cell types, provided that the cells can be identified under a microscope (Fig. 2); this is a valuable advantage when the cells of interest make up only a small fraction of their source tissue, such as maize meiocytes and many other reproductive cells.
- *Cell size*. Maize pollen and meiocytes cannot physically fit through the microfluidic channels of many commonly used single-cell platforms, including the 10× Genomics Chromium (Supplementary Fig. 1). This protocol is not limited by cell size and can handle large cells like meiocytes.
- *Independent sample staging*. This protocol describes how to stage matched samples by microscopy during cell isolation. This makes it possible to directly connect gene expression to known staging by microscopy, a feature that is not easy to implement with current droplet-based scRNA-seq methods.

Other than droplet-based methods, manual single-cell isolation can be performed by using pulled-glass capillary pipettes. This has been applied for single-cell isolation of maize tetrads[12] and *Arabidopsis* egg cells[15]. Capillary isolation is similar to the protocol described here and could be readily incorporated into our pipeline. We have generally found glass pipettes difficult to work with because (i) they break easily and (ii) they are flexible, making it challenging to hold them steadily during cell collection. Metal syringe needles do not suffer from these issues because they are rigid and easier to hold steady.

Comparison with bulk tissue isolation

Several methods for bulk isolation of meiocytes and other plant reproductive cells have been used, including glass capillary pipettes[16], FACS[17] and laser-capture microdissection[18,19]. These often require specialized equipment, large amounts of material and substantial pre-processing before tissue isolation. This protocol is compatible with minute amounts of material (e.g., a single anther) and in many cases may be more practical to implement than bulk approaches. Besides practicality, single-cell methods have an advantage because they can disentangle cell heterogeneity. We previously found that large gene expression changes can occur within classic meiotic stages[3]. Given the potential for within-stage heterogeneity, conclusions from bulk data can be ambiguous (Fig. 3).

Comparison with alternative methods for RNA-seq library preparation and data analysis

We present our current pipeline for single-cell RNA-seq library preparation, which was modified[4] from CEL-seq2 (ref. 5). Alternative methods to construct single-cell libraries in tubes, such as Smart-seq[20,21], would also be fully compatible with this protocol. It is important to choose a library preparation strategy that allows UDIs to mitigate the Illumina index-hopping artefact[8] (see Development of the protocol above).

For data mapping, we provide an example pipeline using the Alevin single-cell RNA-seq package within the Salmon tool[22]. Alevin handles de-multiplexing, UMI quantification and transcript mapping all together, making this tool easier to implement than alternative approaches. Alevin maps to the transcriptome, which in our experience can result in slightly reduced mapping rates because not all transcripts are perfectly annotated in maize; in particular, many transcript annotations do not contain the full-length 3′ untranslated region, which can result in some reads being unmapped in a 3′ tagging library such as those made with CEL-seq. Alternative mapping strategies are described in our previous papers[3,4], but despite these downsides, we recommend starting with Salmon Alevin unless the user is experienced in data analysis.

Limitations

This protocol is effective for the isolation of hundreds of cells with known sample staging but is not practical for the large cell numbers possible with other single-cell methods (e.g., tens of

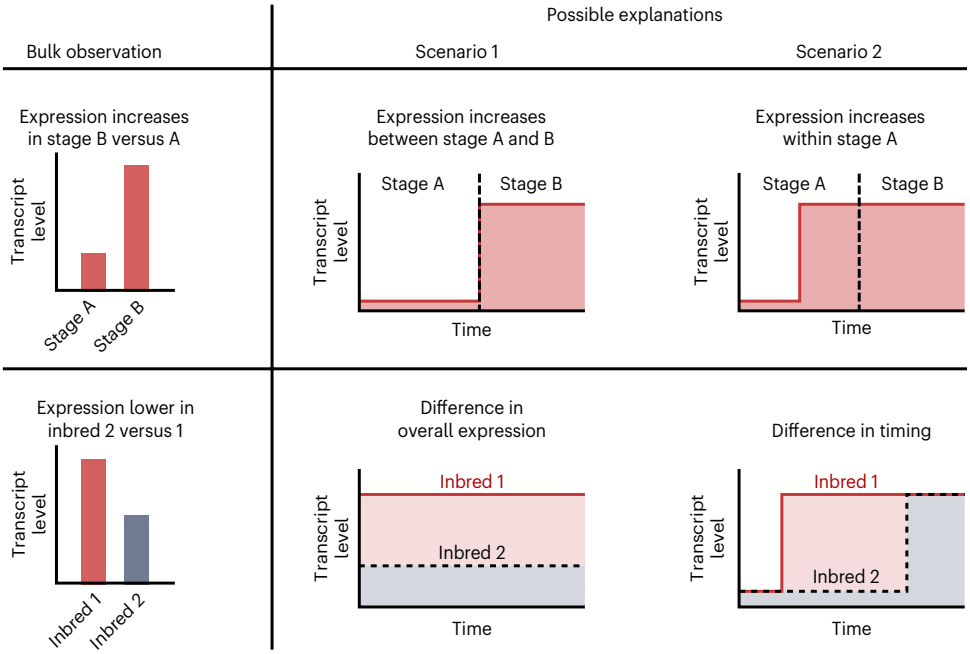

Fig. 3 | Single-cell techniques can resolve cell heterogeneity. This protocol isolates individual cells and pollen precursors, making it possible to resolve cell heterogeneity that would be missed by bulk methods. For example, if an average expression increase were observed between two stages by using bulk methods (top left), it would not be possible to determine if this increase occurred at the stage boundary or within one of the stages. Similarly, if an average expression change were observed when comparing different inbreds or genotypes (bottom left), bulk methods could not distinguish a difference in timing (e.g., a gene was up-regulated later in one inbred) from an expression difference that was consistent over time. Single-cell RNA-seq can resolve these possibilities.

thousands of cells); however, our method specifically isolates rare reproductive cells and so requires much fewer total cells than untargeted approaches. Tissue dissociation methods can vary widely by tissue, and establishing this technique for a new cell type or species will probably require optimization. These RNA-seq libraries are biased for the 3′ end of transcripts, strongly enriched for polyA+ RNA and are not well suited to study alternative splicing, non-polyA transcripts or very short RNAs.

Expertise needed

Manual cell isolation requires fine motor skills similar to those needed for many tissue-dissection protocols. Familiarity with basic epifluorescent microscopy is required for cell staging. Molecular biology skills and training in enzymatic reactions are needed for library preparation. An understanding of command line tools and gene expression analysis is important for analyzing the data; this protocol produces a higher number of reads per cell and allows the collection of independent staging information, making the analysis more similar to that of bulk expression data than that of typical single-cell pipelines in which cell stages must be inferred solely from the expression data. Although the cell-isolation technique can initially appear challenging, it is feasible to learn within a few weeks by using a little practice and patience. Our laboratory has trained a number of researchers, ranging from first-year graduate students to postdoctoral fellows, to collect cells efficiently.

Experimental design

Isolating cells manually

When first learning the isolation technique, begin practicing with samples that are easy to obtain. For example, pollen grains and precursors can be more readily collected than meiotic cells, and so it may be worth starting with pollen grains even if the ultimate goal is to study meiosis.

Your laboratory may also have microspheres in stock, such as sepharose beads or the SPRIselect beads used for genomic DNA isolation. A dilute solution of beads can serve as an excellent training sample to practice moving small structures around with a syringe needle.

Supplementary Video 2 shows the complete cell isolation process in action (Steps 6–12 of the protocol). When learning this technique, it is worth separating the procedure into several stages. First, practice moving the syringe needle tip into the field of view under the microscope. The needle can be moved above the glass slide until a shadow is visible when looking through the microscope eyepieces. After locating the shadow, move the needle downward and rest it gently on the slide. Second, learn to hold the needle tip steady. You do not need the needle to remain perfectly still, but you should be able to hold it within a small area consistently. The needle tip can be rested on the slide to stabilize it, but do not press so hard that the metal becomes bent. Pay close attention to the placement of your hand when holding the syringe. The bottom of your hand (the portion closest to the little finger) should rest on the microscope stage. The syringe should be placed in the palm so that it is stabilized against the hand, with the syringe tip located so that it can be adjusted with either the middle of the index finger or the tip of the thumb. We have found that different users prefer to hold the syringe differently, and when starting out, it is worth trying several positions as you learn what works best for you. See Supplementary Video 2 for an example hand placement.

After becoming comfortable with initial hand placement and syringe needle movements, put a drop of liquid containing practice material (e.g., pollen or beads) on a glass slide. Try picking up and then releasing single pollen grains or beads by using the syringe needle. To collect cells, use your thumb or index finger to move the syringe plunger slightly upward and withdraw the cell into the needle tip. It will take practice to learn the amount of pressure to apply; only small movements of the syringe plunger are necessary. The slide can be moved with your other hand to control the position of the sample. Before placing the needle tip in solution, notice whether there is a droplet of liquid visible at the syringe needle tip. If there is a droplet, it may indicate that there is pressure in the syringe; gently tap the needle tip on an empty part of the glass slide to remove this droplet and equalize the pressure.

Microscopy setup for cell isolation

For single-cell and pollen grain isolation, a basic tissue-culture style inverted microscope will work, and there is no need for a particular manufacturer or build. Important microscope parameters include the following: an inverted microscope (with the objectives below the stage), a flat stage that allows slides to be moved around freely by hand (Supplementary Video 2) and low-numerical aperture (NA) objectives and condensors (e.g., a ×10 objective of NA ≤0.3; a condensor with NA ≤0.3). The low NA allows larger working distances when collecting single cells. For applications beyond meiosis and pollen development, it is possible to adapt this protocol to isolate cells labeled with a fluorescent reporter (e.g., YFP; see Fig. 2, bottom); in such cases, an inverted tissue-culture style microscope with an epifluorescent light source and filters can be used.

Controls for library preparation

During CEL-seq2 library preparation, purified total RNA can be used as a positive control, and an empty tube can be used as a negative (no-template) control. These controls may be particularly valuable when first trying the technique. The positive control can be generated by first making up a solution of 10 ng/ul total RNA (total RNA from any tissue; purified by using standard kits or protocols). Next, add 0.5 µl of this solution to the cap of a PCR eight-tube strip. Add 2 µl of CEL-seq lysis buffer to the cap as described in Step 23 and proceed with Steps 23–30 as normal. For Step 31, do not pool material; instead, add 13.8 µl of SPRIselect beads to the single control sample and proceed with bead purification and in vitro transcription (IVT) as described in Steps 32–41 (with all remaining volumes as normal). The negative control can be generated similarly by adding 2 µl of CEL-seq lysis buffer directly to a clean PCR cap (without a cell). After running on a TapeStation as described in Step 41, the positive control should produce a range of longer amplified RNA (aRNA; Fig. 4b), whereas the negative control should contain only the ~75-nucleotide (nt) background peak (e.g., Fig. 4c).

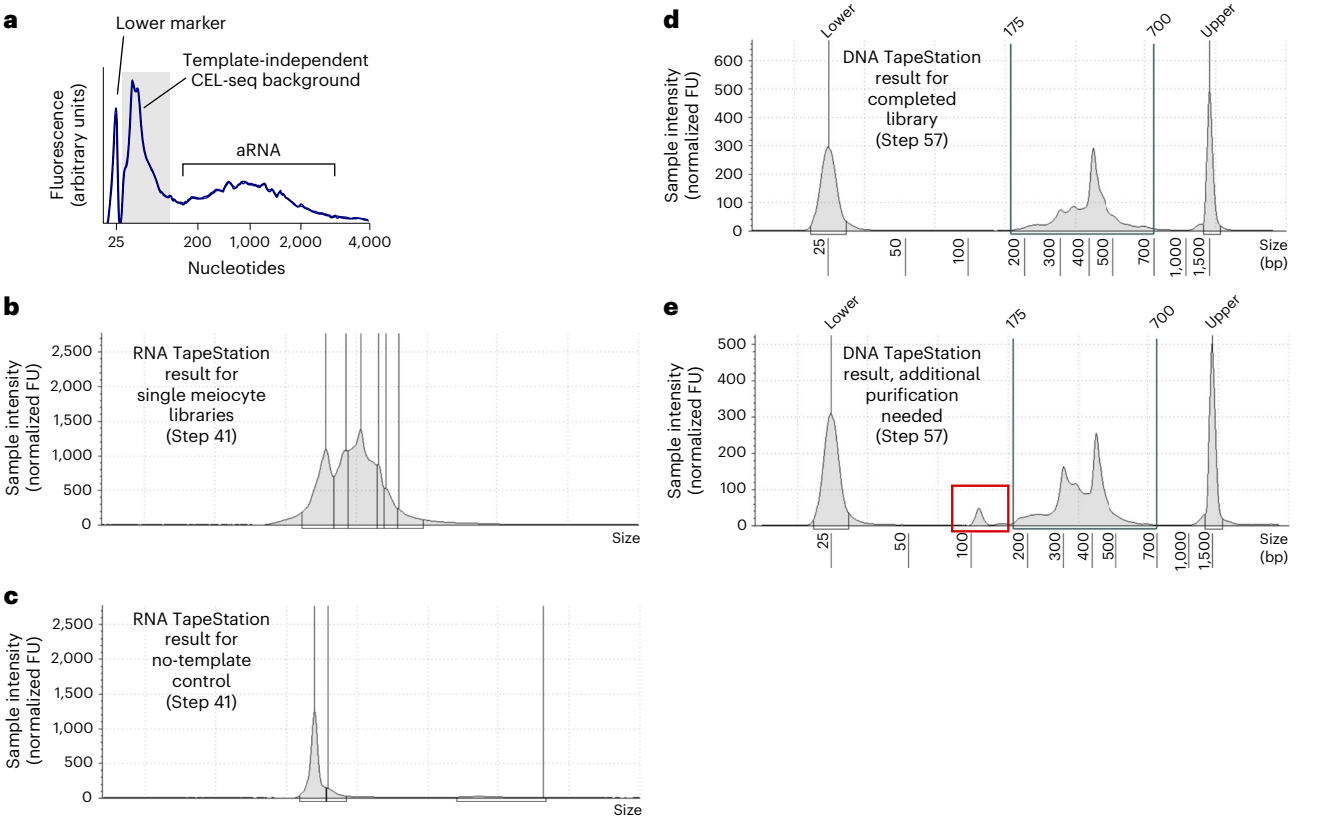

Fig. 4 | Example RNA and DNA size distributions during CEL-seq library preparation. a, aRNA analyzed on an Agilent Bioanalyzer. A successful library shows the lower marker included in the Bioanalyzer loading buffer, a peak around 75 nt that is template independent and then a larger spread-out peak of aRNA. **b**, A successful library measured on the TapeStation. Here, the absolute size distribution was not determined, because the lower 75-nt peak overlapped the lower marker, resulting in the error 'Marker(s) not detected'. Library quality can still be assessed on the basis of the shape of the curve and the presence of a spread-out RNA size to the right of an initial peak. **c**, An example negative control library with only the 75-nt peak. **d**,**e**, TapeStation DNA results showing the final size distribution of single-cell CEL-seq libraries. The library in **e** has density below 200 bp contributed by primer dimers (highlighted in a red box), which can be removed by repeating bead purification. FU, flourescence units.

Sample size considerations

Many single-cell techniques isolate thousands of cells; this can be important when the cell identity must be inferred directly from the expression data, and many distinct cell types are present. This protocol is less reliant on such large cell numbers because it targets specific cell types, and staging information is obtained independently by microscopy. Furthermore, maize meiotic cells and pollen grains have more RNA than a typical somatic cell (correlated with their large size), and so library complexity is high. A key question to consider when choosing the number of cells to collect is how much cell heterogeneity is expected within a sample. We recommend estimating the frequency of the rarest type of cell or pollen grain of interest and collecting enough samples to ensure that this cell or pollen grain type is captured at least 3–10 times. Here are a few examples:

- Pollen grains have active gene expression from their haploid genome. As a result, mutant phenotypes can be directly observed segregating among pollen from a single heterozygous plant. This technique could be used to isolate pollen from a plant that is heterozygous for a mutant allele and determine if the segregating allele caused an expression change in pollen. In such a case, a single time point (mature pollen) might be sufficient, and 12–24 pollen grains would be enough to ensure that multiple pollen grains of each haplotype were isolated.
- scRNA-seq can be used to measure cell cycle-related gene expression from cells that are mitotically dividing but unsynchronized (see, for example, ref. 3). The number of

cells collected is directly related to the temporal resolution of the experiment. If the shortest cell cycle phase of interest is 2 h and a full cell cycle is 24 h, 1 in 12 isolated cells would be expected to be in that phase. To ensure a high chance of recovering at least three cells in the rare cell cycle phase, 96 cells could be collected ($P < 0.05$; binomial distribution).

- For developmental gene expression, the sampling rate needed depends on how quickly gene expression is changing over time, which can be difficult to know before obtaining the data. For new developmental time courses, in practice, we recommend starting with a few hundred cells randomly sampled across time. Then, the data can be analyzed, and additional samples can be obtained if further resolution is required. Example time courses during maize meiosis and pollen development can be seen in refs. 3,4. Given that meiosis can be synchronized between cells of a single anther, it is also worth considering that the cells within an anther are not always independent, and more anthers may need to be collected to ensure sufficient sampling.

Pooling single-cell libraries for sequencing

This protocol enables combinatorial indexing to allow thousands of single-cell libraries to be sequenced together. The first group of indexes is added during reverse transcription in Step 25; this step incorporates the CEL-seq barcodes and allows 48 samples to later be pooled (Fig. 1). Sequences for the CEL-seq primers used during reverse transcription can be found in Supplementary Table 1. The barcode sequences used in these primers are unmodified from the original CEL-seq2 (ref. 5). An example CEL-seq primer, with the barcode and UMI locations annotated, can be found in Supplementary Fig. 2a.

The second step of indexing is during the final PCR (Step 48); this step completes the Illumina adapters and can have up to 384 different unique dual indexes (when using different Integrated DNA Technologies (IDT) xGen primer pairs; see the manufacturer's instructions). The xGen primer pairs are sold in plates, with each well containing a different set of indexed primers (corresponding to a distinct UDI). CEL-seq libraries made with different xGen primer pairs in Step 48 can later be pooled and sequenced together. A diagram of a completed CEL-seq libary fragment is shown in Supplementary Fig. 2b, with the internal CEL-seq barcode and Illumina indexes labeled.

Materials

Biological materials

- *Zea mays* L. subsp. *mays* cultivar 'W23 bz2 Walbot' (US National Plant Germplasm accession: PI 674441)

Reagents

Tissue dissociation and microscopy staging

- Liquid nitrogen (AirGas)
  ▲ **CAUTION** Liquid nitrogen is a skin and eye irritant. Avoid contact with skin and eyes. Handle by using appropriate protective equipment.
- 100% ethanol (EtOH), absolute (200 proof) (Fisher Scientific, cat. no. BP2818-500)
  ▲ **CAUTION** EtOH is flammable. It is a skin and eye irritant. Avoid contact with skin and eyes. Handle by using appropriate protective equipment.
- Glacial acetic acid (Sigma-Aldrich, cat. no. 27225-500ML-R)
- Ultrapure DNase/RNase-free distilled water (Invitrogen, cat. no. 10977015)
- PBS, pH 7.4, sterile filtered, suitable for cell culture (Sigma-Aldrich, cat. no. 806552)
- DAPI (Sigma-Aldrich, cat. no. D9542-10MG)
- Trehalose dihydrate (PhytoTechnology Laboratories, cat. no. T7968)
- MES hydrate (Sigma-Aldrich, cat. no. M2933)

Library preparation

- Triton X solution (Sigma-Aldrich, cat. no. 93443)
  ▲ **CAUTION** Triton X is a skin and eye irritant. Avoid contact with skin and eyes. Handle by using appropriate protective equipment.
- PEG-8000 (Sigma-Aldrich, cat. no. 25322-68-3)
- 2.5 M NaCl (Sigma-Aldrich, cat. no. 7647-14-5)
- SPRIselect reagent kit (Beckman Coulter Life Sciences, cat. no. B23317)
- RNAClean XP reagent kit (Beckman Coulter Life Sciences, cat. no. A63987)
- Agilent TapeStation RNA high-sensitivity buffer (Agilent, cat. no. 5067-5580)
- Agilent TapeStation RNA high-sensitivity screentape (Agilent, cat. no. 5067- 5579)
- Agilent TapeStation DNA high-sensitivity buffer (Agilent, cat. no. 5067-5603)
- Agilent TapeStation DNA high-sensitivity screentape (Agilent, cat. no. 5067-5584)
- Invitrogen Superscript IV reverse transcriptase, 200 U/µl (Life Technologies, cat. no. 18090200)
- NEB Exonuclease I, 20,000 U/ml (New England Biolabs, cat. no. M0293L)
- NEB 10 mM dNTP mix (New England Biolabs, cat. no. N0447L)
- NEBNext Ultra II non-directional RNA second strand synthesis module (New England Biolabs, cat. no. E6111)
- HiScribe T7 high-yield RNA synthesis kit (New England Biolabs, cat. no. E2040S)
- NEB Protoscript II reverse transcriptase (New England Biolabs, cat. no. M0368L)
- USER enzyme (New England Biolabs, cat. no. M5505S)
- Q5 Ultra II NEBNext 2× master mix (New England Biolabs, cat. no. M0544L)
- Barcoded CEL-seq primer plate (Supplementary Table 1; IDT, custom Ultramer DNA oligonucleotides; order specifications: 4 nmol of Ultramer oligo, TruGrade, machine-mixed bases, standard desalting, normalized to 50 µM in IDTE buffer pH 8.0, ship on dry ice)
- hpN6 DNA oligo (5′-GATCGGAAGT/ideoxyU/ ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNN-3′; IDT, custom DNA oligonucleotide; order specificiations: 250 nmol, standard desalting, machine-mixed bases)
- Illumina P5 DNA oligo (5′-AATGATACGGCGACCACCGA-3′; IDT, custom DNA oligonucleotide; order specificiations: 100 nmol, standard desalting)
- Illumina P7 DNA Oligo (5′-CAAGCAGAAGACGGCATACG-3′; IDT, custom DNA oligonucleotide; order specificiations: 100 nmol, standard desalting)
- IDT xGen UDI primers (IDT, cat. no. 10005975 or 10005922)
- Aluminum sealing foil, 96-well plate size (McKesson, cat. no. 1017527)

Equipment

- Stage micrometer, 2.0 mm (Ward's Science, cat. no. 94-9910)
- 1.7-ml Posi-Click graduated microcentrifuge tubes, boilproof, clear (Thomas Scientific, cat. no. 1149K01)
- 0.1-ml eight-strip tubes, low profile, with separate eight-strip clear flat caps, natural (Globe Scientific, cat. no. PCR-STR-01F)
  ▲ **CRITICAL** We recommend using this supplier for PCR caps if possible. We have found that optical caps from different suppliers can vary in clarity and surface properties that can affect the ease of cell release and visibility under the microscope.
- Disposable Petri dishes (VWR, cat. no. 25384-302)
- Pre-cleaned glass microscope slides (Thomas Scientific, cat. no. 1201A70)
- 135-mm fine-tip straight long tweezers (Towot, part no. SA-12)
- Scalpel handle, no. 3, stainless steel, 5 inches (Thomas Scientific, cat. no. 3883H10)
- Blade, #11, sterile, carbon blade (Thomas Scientific, cat. no. 2001T11)
- 1-ml Luer slip syringes (CareTouch, cat. no. CTSLS1)
- 32-gauge blunt-tip dispensing needle (BSTEAN, cat. no. DN-05-YL-32)
- Dissecting stereo microscope (VWR, cat. no. 10836-004)
- Olympus IX70 inverted microscope (Olympus, cat. no. OLY-IX70-PFL-R)
- Veriti 96-well fast thermal cycler (Applied Biosystems, cat. no. 4375305)

- 4150 TapeStation system (Agilent, cat. no. G2992AA)
- 96-well magnetic rack (Sergi Lab Supplies, Amazon cat. no. B08134P9RT)
- 1.5-ml magnetic rack (Sergi Lab Supplies, Amazon cat. no. B0812XLPVK)

Software and packages

- RStudio V2022.12.0+353 (https://posit.co/downloads/)
- Salmon/1.9.0-gompi-2020b (https://github.com/COMBINE-lab/salmon)
- Tximport V1.0.3 (https://bioconductor.org/packages/tximport/)

Reagent setup

hpN6 primer

Resuspend at 1 mM with nuclease-free water. After resuspending, store at −20 °C for ≤2 years.

Illumina P5 and P7 primers (optional)

P5 and P7 primers are used to amplify libraries if the concentration is too dilute. Resuspend each primer to 100 µM with nuclease-free water. After resuspending, store at −20 °C for ≤2 years.

Prechilled empty 100-µl pipette tip boxes

For storing eight-tube strips after single-cell collection, save empty 100-µl pipette tip boxes and place at −80 °C to prechill.

Ethanol acidic acid (EAA) fixative

Combine three parts of 100% EtOH to one part glacial acetic acid. Prepare EAA fixative the same day as collection.

Pollen isolation buffer

Prepare buffer with 400 mM trehalose and 4 mM MES, pH 5.7. Pollen isolation buffer can be stored at 4 °C for 1 month or frozen in 1-ml aliquots and stored at −20 °C.

Glycerol mounting medium

Combine 9 parts of 100% glycerol and 1 part of 1× PBS. This medium can be stored at room temperature (22 °C) for 1–2 weeks.

DAPI stain

Prepare 5 mg/ml (500×) stock in nuclease-free water. Store at −20 °C protected from light for ≤5 years. Thaw stock DAPI and dilute 1 part of stock DAPI in 499 parts of 1× PBS. Prepare the dilution the same day of staining.

CEL-seq lysis buffer plate

Add 1.5 µl each barcoded CEL-seq primer to a separate well of a 96-well plate. To each well, add 7.5 µl of 10 mM dNTP mix, 1.1 µl of 10% Triton X-100 and 89.9 µl of nuclease-free water. Mix by pipetting up and down. For storage, seal the plate with an adhesive foil. This stock can be stored at 80 °C for ≤2 years and thawed shortly before use.

DNA bead binding buffer

Add 2 g of PEG-8000, 5 ml of NaCl (5 M stock) and 2 ml of RNase-free water to a 15-ml conical tube. Shake gently for 1 h with occasional vortexing until PEG-8000 is dissolved. Fill the tube with RNase-free water up to the 10-ml line. Store at 4 °C for ≤6 months. Remove the tube from the refrigerator and allow it to come to room temperature 30 min before use.

Creating reference transcriptome file (cDNA fasta)

Download the reference transcriptome file (cDNA fasta) for the current maize genome build. For the B73 v5 genome, this can be found at https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/902/167/145/GCF_902167145.1_Zm-B73-REFERENCE-NAM-5.0/GCF_902167145.1_Zm-B73-REFERENCE-NAM-5.0_rna.fna.gz.

Installation of Salmon packages

The version of the package used in this analysis is Salmon/1.9.0-gompi-2020b. To install and activate Salmon, use the following command:

```
$ conda config –add channels conda-forge
$ conda config –add channels bioconda
$ conda create -n salmon salmon
$ conda activate salmon
```

Example data

The 'Data analysis' section can be followed by using example data from ref. 3, which can be accessed on the Sequence Read Archive under accessions SRR7989735 and SRR7989736.

Procedure

Maize anther collection and tissue dissociation

● **TIMING 2 h 30 min**

▲ **CRITICAL** Prepare EAA fixative in advance and keep it on ice for this protocol.

1. Collect tassel(s) with outer leaf sheaths intact and wrap them in a damp paper towel. This will prevent the tassel from prematurely drying out.
2. Add 100 µl of EAA fixative into each tube of an eight-tube strip and label the strip. Place tubes on ice.
3. Remove a spikelet from the tassel with forceps and place it onto a microscope slide.
4. View the spikelet on a stereomicroscope. Cut the spikelet at the base by using fine-tip forceps and then use the forceps to apply pressure from the top (uncut) portion of the spikelet to push out the anthers (Fig. 1). Each spikelet contains an upper and lower floret with three anthers each; after dissection, anthers from the upper floret can be distinguished because they are larger (Fig. 1). Select the anthers from the upper floret and measure the length of one anther with a stage micrometer. Record anther length so it can later be associated with the corresponding scRNA-seq data. See Supplementary Table 2 for guidelines on the relationship between maize anther length and developmental stage.
   ▲ **CRITICAL STEP** Be careful not to damage the anthers during dissection. Proceed quickly to the following step.
5. Meiotic-stage cells can be isolated from 1.0–2.5-mm anthers by following option A; pollen grains and post-meiotic pollen precursors can be isolated from ≥2.5-mm anthers by following option B.
   (A) **Tissue dissociation to release meiotic-stage cells**
      (i) Place three anthers from the floret into a tube of fixative. Incubate the fixed anthers on ice for 2–5 h.
      (ii) While the anthers are incubating, repeat Steps 1–4 to collect more samples from additional spikelets.
      (iii) Remove the EAA fixative from the tubes by pipetting without disturbing the anthers.
      (iv) Add 100 µl of pre-chilled 0.1× PBS. Allow the anthers to rehydrate on ice for 5 min.
      (v) Carefully remove one anther from the tube and place it onto a clean microscope slide.
      (vi) Using a scalpel, cut the anther in half (Supplementary Video 1).
      (vii) Add 20 µl of sterile 0.1× PBS onto the slide and press the anther with the scalpel to release cells (Supplementary Video 1). Continue immediately to Step 6 for single-cell isolation.
      ■ **PAUSE POINT** Cell collection for scRNA-seq must be completed immediately, but the remaining fixed anthers can be stored at 4 °C for ≤4 weeks for microscopic staging.
      ◆ **TROUBLESHOOTING**

(B) **Tissue dissociation to release pollen grains and pollen precursors**

● **TIMING 2 h 30 min**

(i) Place two anthers from the floret into a tube of fixative to save for microscopic staging (Steps 17–21). Place the third (unfixed) anther onto a clean microscope slide in a drop of 20 µl of pollen isolation buffer.

■ **PAUSE POINT** The two fixed anthers can be stored in fixative at 4 °C for ≤4 weeks for independent stage verification. For the third anther, pollen collection for RNA-seq must be completed immediately.

(ii) Cut the anther in half by using a scalpel. Press the anther with the scalpel to release the pollen grains or pollen precursors (Supplementary Video 1). Proceed immediately to Step 6.

◆ **TROUBLESHOOTING**

Isolation of single-cells, pollen precursors or pollen grains

● **TIMING 5–10 min per each set of eight cells**

▲ **CRITICAL** In the steps below, we use the term 'cells' for simplicity. During meiosis and early post-meiotic stages (e.g., unicellular microspores), individual cells are collected. For late stages of pollen development, the same proceedure is followed, but it should be understood that the structure is multicellular. In these cases, the protocol is used identically to isolate individual bicellular microspores or tricellular pollen grains.

6. Fill a fresh 1-ml syringe to 0.3–0.5 ml of medium (0.1× PBS for meiotic stages and pollen isolation buffer for post-meiotic stages) and then place a fresh 32-gauge blunt-end needle on the tip and expel the air.
7. Place an eight-strip of PCR caps face up on a microscope slide. Move this slide out of the way toward the back of the microscope stage. There should now be two slides on the microscope: a slide with released cells (from Step 5) located over the objective for visualization, and a second slide with PCR caps located toward the back of the stage and ready for cell collection.
8. Under the microscope at 10× magnification, locate a meiotic cell or pollen precursor on the basis of morphology (Fig. 2). Meiotic cells are much larger than surrounding somatic cells and have a granular cytoplasm (Fig. 2, top). Pollen precursors are spherical (Fig. 2, middle) and increase in size during development.
9. Place the syringe needle tip next to the cell. To find the needle under the microscope, move the tip above the surface of the liquid until the shadow is visible within the field of view. Then, gently lower the tip and rest it near the cell.
10. Gently withdraw the syringe by using light pressure to pick up the cell. Watch the cell under the microscope during this process to confirm that the cell has been picked up (Supplementary Video 2).

    ▲ **CRITICAL STEP** It is ok to pick up neighboring cells during this step, because they will be removed during the wash step below.

    ◆ **TROUBLESHOOTING**
11. To wash the cell, move the syringe needle to a clean portion of the slide and gently press down on the syringe to eject the cell along with a droplet of medium. This will reduce cell density and debris to make collection of a single cell easier. Find the target cell again under the microscope and pick it up as described in Steps 8–10.

    ▲ **CRITICAL STEP** Release enough liquid so that the target cell is well separated from others.
12. Move the slide with PCR caps into the field of view and release the cell into a small droplet onto the inside of one cap. Confirm cell release visually under the microscope.

    ◆ **TROUBLESHOOTING**
13. Repeat Steps 8–12 to isolate seven additional cells, filling the eight-cap strip.

    ▲ **CRITICAL STEP** Work quickly to prevent the cells from drying out on the caps. With practice, each set of eight cells can be collected within 5–10 min.
14. Once eight cells are collected, label the tubes of an eight-tube PCR strip with a sample ID. Then, carefully place the tubes onto the caps to seal, keeping the caps face up. Place the capped tubes into liquid nitrogen to flash freeze them (Supplementary Video 3).

▲ **CRITICAL STEP** Be mindful to not disturb the droplet in the cap during this process. The droplet will remain in the cap during flash freezing and should stay there until lysis (Step 23).

■ **PAUSE POINT** The tubes with collected cells can sit in liquid nitrogen until finished with all collections of the day.

15. Repeat collections for additional anthers. For meiotic stage cells, additional anthers will have already been placed in fixative in Step 5A(ii), and cells can be collected from these anthers by following Step 5A(iii–vii) and then Steps 6–14. For pollen grains and pollen precursors, fresh anthers should be isolated by following Steps 1–4 and 5B, and then single pollen grains and precursors can be isolated by following Steps 6–14.
16. After all collections are completed, remove the tubes from liquid nitrogen and place them in a pre-chilled, empty 100-µl pipette tip box in a −80 °C freezer.

■ **PAUSE POINT** The samples can now be stored at −80 °C for ≤6 months until ready to proceed with library preparation.

Microscopy staging using DAPI

● **TIMING 2 h**

▲ **CRITICAL** Fixed samples can be stored at 4 °C for ≤4 weeks, allowing staging to be completed at a different time from cell isolation.

17. Remove a single fixed anther collected in Step 5A(i) or 5B(i) and place it on a clean microscope slide. These anthers are from the same spikelet as those used for cell collection and can provide matched staging information. Slice the anther in half with a scalpel and then add 20 µl of DAPI stain (10 µg/ml; see Reagent setup).
18. Press the anther halves against the microscope slide by using the scalpel (with the cutting surface of the scalpel facing up) to release cells or pollen grains. Incubate protected from light for 30 min.
19. Place one drop (~10 µl) of glycerol mounting medium on the slide and place a coverslip on top.
20. Remove excess glycerol with a Kimwipe and apply nail polish to the edges of the coverslip to seal.

■ **PAUSE POINT** Once slides are prepared, they can be stored at 4 °C for 2–7 d.

21. Visualize the slides by using a fluorescent microscope with a ×40 or ×63 oil objective and DAPI filters and identify developmental stages (see refs. 3,4,23 for guidance).

Library preparation

● **TIMING 2 d**

▲ **CRITICAL** The following mixes have been calculated to make 48 single-cell libraries.

Cell lysis

● **TIMING 20 min**

22. Remove the eight-tube strips with collected cells from Step 16 from the freezer and place them on ice.
23. Add 2 µl of CEL-seq lysis buffer onto the inside of the cap of the eight-tube strip, on top of the position where the isolated cells or pollen grains were placed. Gently close the PCR tubes and invert them so that the cap is face up with the liquid drop still on the inside of the cap (Supplementary Video 3).

▲ **CRITICAL STEP** Use a different well of the CEL-seq lysis buffer plate for each cell. The lysis buffer includes barcoded CEL-seq primers (see Reagent setup), and each well has primer with a different barcode sequence. This allows samples to be pooled in Step 31 and then distinguished computationally after sequencing.

■ **PAUSE POINT** After adding lysis buffer, the tubes can be placed back into the −80 °C freezer and processed further on another day.

Reverse transcription through second strand synthesis

● **TIMING 3 h 30 min**

24. For reverse transcription of cellular RNA, prepare reverse transcription (RT) Mix I according to the table below:

Component	Amount (µl)	Final concentration in master mix	Final concentration when added to sample
Superscript IV reverse transcriptase (SSIV) buffer	36	3×	1×
DTT	9	15 mM	5 mM
H_2O	10.5	–	–
Superscript IV reverse transcriptase enzyme	4.5	15 U/µl	5 U/µl

▲ **CRITICAL STEP** Thaw all reagents in advance and keep assembled RT Mix 1 on ice.

25. Incubate the tubes in a thermal cycler at 65° with the heated lid set to 70 °C. After 2 min, remove the tubes from the thermal cycler, vortex with the caps face down and briefly centrifuge the tubes to collect the cell lysate in the bottom of the tubes. Incubate at 65 °C for an additional 3 min and immediately place the tubes on ice for 1–5 min (Supplementary Video 3).

▲ **CRITICAL STEP** Be sure that the heated lid is at 70 °C for this step.

26. Add 1 µl of RT Mix 1 to each sample. Mix the reaction by a brief vortex and spin down samples to collect liquid in the bottom of the tube. Incubate the samples in a thermal cycler under the following conditions with the heated lid set to 70 °C. Place the samples on ice before moving to the next step.

Cycle number	Condition
1	42 °C, 2 min
2	50 °C, 15 min
3	55 °C, 10 min

27. To digest residual CEL-seq primers, prepare the following Exo1 master mix:

Component	Amount (µl)	Final concentration
NEB Exonuclease 1 buffer	3	1×
RNase-free water	24	–
NEB Exonuclease 1	3	2 U/µl

28. Add 0.5 µl of Exo1 master mix to each sample. Place the samples in a thermal cycler and incubate according to the following table with the heated lid set to 105 °C. After incubation, place the samples on ice.

Cycle number	Condition
1	37 °C, 20 min
2	80 °C, 10 min

29. To convert cDNA to double-stranded DNA, prepare a second strand synthesis mix for all reactions:

Component	Amount (µl)	Final concentration in master mix	Final concentration when added to sample
NEBNext Ultra II non-directional RNA second strand synthesis buffer	60	1.4×	1×
RNAse-free water	327.4	–	–
NEBNext Ultra II non-directional RNA second strand synthesis enzyme	30	1.4×	1×

30. Add 8 µl of second strand synthesis mix to each tube. Incubate for 2 h at 16 °C in a thermal cycler with no heated lid.

SPRIselect bead purification

● **TIMING 1 h 30 min**

31. Pool 48 samples in a fresh 1.7-ml tube (~550 µl in total). Add 30 µl of SPRIselect beads and 635 µl of bead binding buffer. Incubate the tube at room temperature for 15–30 min.
▲ **CRITICAL STEP** Allow the SPRIselect beads and bead binding buffer to come to room temperature for 30 min before use. Only pool samples that were made with different CEL-seq primers.
32. Place the tube on a magnet for 20 min or until the solution is clear. While the tube is still on the magnet, carefully remove the liquid by pipetting without disturbing the beads and discard the liquid.
33. To wash the beads, add 100 µl of 80% EtOH (vol/vol) to the tube. While the tube is still on the magnet, carefully remove the liquid without disturbing the beads and discard the liquid. Repeat with a second wash and then allow the beads to dry for 15 min or until the beads lighten in color and all visible EtOH is gone.
34. Remove the samples from the magnet. Add 6.4 µl of RNAse-free water and pipette the samples up and down until the beads are resuspended. Incubate at room temperature for 2 min. The beads can remain in solution during the following step of IVT.

IVT

● **TIMING 10–16 h**

35. Prepare the IVT mix by combining the following:

Component	Amount (µl)	Final concentration in master mix	Final concentration when added to sample
UTP (100 mM)	2	16.7 mM	10 mM
ATP (100 mM)	2	16.7 mM	10 mM
GTP (100 mM)	2	16.7 mM	10 mM
CTP (100 mM)	2	16.7 mM	10 mM
T7 polymerase buffer	2	1.7×	1×
T7 polymerase enzyme	2	1.7×	1×

36. Add 9.6 µl of IVT mix to the tube from Step 34. Incubate overnight (10–16 h) at 37 °C.

aRNA purification

● **TIMING 1 h 25 min**

37. Place the tube from IVT (Step 36) on a magnet for 2 min or until the solution is clear. While the tube is still on the magnet, transfer liquid to a fresh PCR tube by pipetting.
38. Add 28.8 µl of RNAClean beads to the tube and pipette up and down to mix. Incubate at room temperature for 15 min. Place the tube on a magnet for 5 min or until the solution is clear. With the tube still on the magnet, carefully remove the supernatant by pipetting without disturbing the beads and discard the supernatant.
▲ **CRITICAL STEP** Allow RNAClean XP beads to come to room temperature for 30 min before use.
39. Wash the beads two times with 100 µl of 85% EtOH (vol/vol). Remove EtOH by pipetting and allow the beads to dry for 15 min or until the beads lighten in color and no drops of ethanol are visible.
40. Add 6.5 µl of RNAse-free water to the tube and pipette up and down to mix. Incubate for 2 min at room temperature. Place the tube on ice.

RNA quality check

● **TIMING 45 min**

41. Place the sample on a magnet to move the beads to the side. Remove 2 µl and run on an Agilent TapeStation high-sensitivity RNA chip. Move the tube with aRNA back to ice. CEL-seq libraries result in a template-independent peak around 75 nt (Fig. 4a,c). On the TapeStation, this peak can overlap the lower marker used to align each lane; as a result, it is common

to get the error 'Marker(s) not detected' and see no nucleotide sizes associated with the data. This does not indicate a problem, and libraries can be assessed on the basis of the size distribution even without knowing the absolute RNA sizes: a successful library has a small early peak, followed by continuing density to the right (Fig. 4b shows an example successful library when the 'Marker(s) not detected' error appeared; compare to the no-template control in Fig. 4c).

▲ **CRITICAL STEP** Proceed immediately to Step 42 after loading the TapeStation.

◆ **TROUBLESHOOTING**

Reverse transcription 2

● **TIMING 2 h**

42. Prepare priming mix by combining the following components:

Component	Amount (µl)	Final concentration in priming mix	Final concentration during RT (Step 45)
dNTPs (10 mM each)	2.5	3.3 mM each	0.5 mM each
Nuclease-free water	4.5	–	–
hpN6 primer (1 mM)	0.5	66.7 µM each	10 µM

43. Prepare RT Mix 2 by combining the following components:

Component	Amount (µl)	Final concentration in RT Mix 2	Final concentration during RT (Step 45)
DTT (100 mM)	1.6	25 mM	10 mM
Protoscript II buffer	3.2	2.5×	1×
Nuclease-free water	0.8	–	–
Protoscript II enzyme	0.8	25 U/µl	10 U/µl

44. Add 1.5 µl of priming mix to the tube from Step 41. Incubate for 5 min at 65 °C with the heated lid set to 85 °C. Chill on ice for >1 min.
45. Add 4 µl of RT Mix 2 to the tube. Incubate under the following conditions with the heated lid set to 85 °C:

Cycle	Condition
1	25 °C, 10 min
2	42 °C, 1 h
3	70 °C, 10 min

46. Add 0.5 µl of USER enzyme to the tube. Incubate at 37 °C for 30 min.

■ **PAUSE POINT** Samples can be stored at −80 °C for ≤1 year until ready to proceed with PCR.

PCR amplification

● **TIMING 1 h**

47. Prepare PCR mix by combining the following components:

Component	Amount (µl)	Final concentration
IDT xGen primers	5.5	1 µM each
Sample from Step 46	3	–
Nuclease-free water	23.2	–
Q5 Ultra II NEB master mix	23.2	1×

▲ **CRITICAL STEP** The xGen primers come with multiple different Illumina index combinations. For libraries that will be pooled and sequenced together, it is important to use primers with different indexes (which are found in distinct wells on the IDT xGen plate; see the manufacturer's instructions).

48. Run PCR in a thermal cycler under the following conditions:

Cycle number	Denature	Anneal	Extend	Hold
1	98 °C, 30 s	–	–	–
2–16	98 °C, 10 s	65 °C, 30 s	72°, 30 s	–
17	–	–	72 °C, 5 min	–
18	–	–	–	10 °C

Bead purification and size selection

● **TIMING 3 h**

49. Add 50 µl of SPRIselect beads to the PCR product. Pipette up and down to mix. Incubate the tube at room temperature for 15 min.
    ▲ **CRITICAL STEP** Allow SPRIselect beads to come to room temperature for 30 min before use.
50. Repeat Steps 32 and 33 to wash the beads with EtOH and allow the washed beads to dry.
51. Add 40 µl of RNAse-free water to the tube and pipette up and down to resuspend the beads. Incubate at room temperature for 2 min.
52. Add 24 µl of SPRIselect beads and pipette up and down to mix. Incubate for 15 min at room temperature.
53. Place the tube on the magnet for 5 min or until the solution is clear. Carefully transfer 60 µl of the supernatant to a fresh PCR tube, being careful not to withdraw any beads.
54. Add 7.5 µl of SPRIselect beads to the supernatant tube and pipette up and down to mix. Incubate the tube at room temperature for 15 min.
55. Place the tube on the magnet for 5 min or until the solution is clear. Remove the supernatant and wash two times with 80% EtOH (vol/vol). Remove EtOH with the tube on the magnet. Allow the beads to dry for 15 min or until the beads lighten in color and all drops of EtOH are gone.
56. Add 20 µl of RNAse-free water to the tube to elute DNA. Pipette up and down to mix and incubate the tube for 2 min at room temperature. Place the tube on the magnet for 5 min or until the solution is clear. Remove 20 µl of solution and transfer to a fresh tube.
    ■ **PAUSE POINT** Samples can be stored at −80 °C for ≤1 year until ready to proceed with library quality control and sequencing.

Library quality check

● **TIMING 45 min**

57. Check the size distribution of the DNA by running a 1-µl sample by using an Agilent TapeStation high-sensitivity DNA chip. The DNA should be between 200 and 800 base pairs (bp) with a peak around 300–400 bp. The desired DNA concentration is >10 nM.
    ▲ **CRITICAL STEP** Density below 200 bp (e.g., Fig. 4e) indicates adapter dimers that can outcompete longer fragments when sequenced; if density in this size range is observed, size selection should be repeated (see Troubleshooting). Multiple libraries can be pooled at this point before sequencing, provided they were made with different xGen primer sets. See standard guidelines[24] for library pooling and normalization; the libraries should be diluted to the same concentration and then mixed in equal proportions.
    ◆ **TROUBLESHOOTING**

Library sequencing

● **TIMING 2 d**

58. Perform paired-end sequencing according to the manufacturer's instructions on an Illumina instrument (e.g., HiSeq 4000 or NovaSeq) and with the TruSeq indexing primers. Paired-end 75 nt is sufficient for these libraries, but longer reads can be used. We aim for a sequencing depth of ~2 million reads per cell.

Data mapping and transcript quantification

● **TIMING 2 d**

▲ **CRITICAL** This section describes how to map and quantify CEL-seq data from ref. 3 by using Salmon Alevin. We have updated the CEL-seq adapter sequences since this earlier publication.

To analyze new data generated with our updated protocol, make the adjustments described in the Critical step sections below.

59. Create a Salmon transcriptome index by running the command below:

```
salmon index -t GCF_902167145.1_Zm-B73-REFERENCE-NAM-5.0_rna.fna.gz -i
transcriptome_index
```

60. To map to the transcriptome, run Salmon Alevin by using the following command line:

```
salmon alevin -l ISR –whitelist CELseq_whitelist_legacy.txt –
umi-geometry 1[1-10] –bc-geometry 1[11-18] –read-geometry 2[7-end]
-i transcriptome_index –tgMap TGMap.tsv -1 SRR7989735_1.fastq.gz -2
SRR7989735_2.fastq.gz -o SRR7989735_output
salmon alevin -l ISR –whitelist CELseq_whitelist_legacy.txt –
umi-geometry 1[1-10] –bc-geometry 1[11-18] –read-geometry 2[7-end]
-i transcriptome_index –tgMap TGMap.tsv -1 SRR7989736_1.fastq.gz -2
SRR7989736_2.fastq.gz -o SRR7989736_output
```

▲ **CRITICAL STEP** The whitelist file (CELseq_whitelist_legacy.txt) and a transcript-to-gene map file for the maize B73 v5 genome (TGMAP.tsv) are provided as Supplementary Data 1 and 2, respectively. To analyze new data generated with this protocol, use the 'CELseq_whitelist.txt' file (Supplementary Data 3) rather than the legacy whitelist. Provide the new read 2 fastq file to the -1 argument and the new read 1 fastq file to the -2 argument. For example, replace '-1 SRR7989736_1.fastq.gz -2 SRR7989736_2.fastq.gz' in the line above with '-1 YourRead2FastqFile.fastq.gz -2 YourRead2FastqFile.fastq.gz'.

61. After running Salmon Alevin, import the data into R by using the tximport package with the following commands:

```
library(tximport)
txi_SRR7989735 <- as.matrix(tximport::tximport("SRR7989735_output/
alevin/quants_mat.gz", type = "alevin")$counts)
txi_SRR7989736 <- as.matrix(tximport::tximport("SRR7989736_output/
alevin/quants_mat.gz", type = "alevin")$counts)
UMI_Count_Matrix <- cbind(txi_SRR7989735, txi_SRR7989736)
```

▲ **CRITICAL STEP** To analyze new data, replace the file directories above (e.g., 'SRR7989735_output') with the directories relevant to your study.

62. Remove cells with low-quality data. We generally exclude cells with <10,000 UMIs or <2,000 expressed genes, as follows:

```
UMI_Counts_Filtered <- UMI_Count_Matrix[, (colSums(UMI_Count_Matrix)
>= 10000) & (colSums(UMI_Count_Matrix > 0) >= 2000)]
```

63. Normalize the data to transcripts per million (TPM) and then log transform them by using a pseudocount, as follows. This log transformed data can then be used for downstream analysis (differential gene expression, plotting expression in a heatmap, etc.). Note: if using the DESeq2 package for differential expression, the UMI_Counts_Filtered matrix should be supplied instead, because this package expects unnormalized data.

```
TPM <- sweep(UMI_Counts_Filtered, 2, colSums(UMI_Counts_Filtered),
"/")*10^6
LogData <- log(TPM + 100, 2)
```

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
5A(vii) and 5B(ii)	Low cell release	Early meiotic stages (<1.5-mm anthers) and certain mutants can be more difficult to release from fixed anthers	Apply additional mechanical force. When cells are difficult to release, an alternative method to apply force is as follows. Apply a piece of Scotch tape to both ends of a glass slide as a spacer. Place this slide on top of the slide with the sample to apply gentle pressure. Move both glass slides back and forth to apply shear force. Lift up one slide and gently rinse any material onto the other slide by using a pipette
10	Cells stick to the glass slide	Cells in early meiotic stages can adhere slightly to the glass slide	Dislodge the cells by gently flowing liquid over them by using a syringe. When cells lift up and are moving, they can then be picked up. Working more efficiently after cell release can also reduce the amount of time that cells have to stick to the glass
12	The target cell cannot be seen on the PCR cap	No cell was released	Practice moving cells around the microscope slide until you are able to reliably release single cells in a small droplet
		The cell is at the edge of the droplet, where it is difficult to see	The released cell may be in the edge of the droplet, where it is difficult to see. Try touching the syringe needle to the top of the droplet and move it slightly; the needle will drag the drop with it so that the cell is no longer obscured by the droplet edge
		The microscope is not in focus	The PCR caps are higher than the original microscope slide and can require refocusing. If no cell is seen, try focusing on the edge of the release droplet
41	The aRNA sample contains no product or only a single early peak (e.g., Fig. 4c)	Mistakes during library preparation, poor quality RNA in cells or lack of collected cells	If no RNA is visible (including the shorter template-independent peak as shown in Fig. 4c), it generally indicates that a reagent was left out during library preparation or may mean that an enzyme has expired. Repeat library preparation with fresh reagents
			If only a single early peak is visible, it generally indicates that the quality of RNA in the collected cells has been degraded. Keep reagents on ice when possible, work quickly during cell isolation and ensure that cells are visible in the PCR tube caps
57	The sample contains small fragments (<200 bp)	Size selection did not remove all small fragments	Repeat bead purification by using a 0.8× ratio of SPRIselect beads (see the manufacturer's instructions)
	The sample shows large fragments (>800 bp)	Size selection did not remove all large fragments	Repeat size selection to remove large fragments with SPRIselect beads (see the manufacturer's instructions)
	Concentration <10 nM	Material was lost during size selection or not amplified sufficiently during PCR	If no DNA is visible, repeat PCR from Steps 47 and 48 by using excess RT Mix 2 (from Step 46)
			If the DNA concentration is too low, run the sample for 5–10 additional cycles of PCR as described in Steps 47 and 48 except instead of the xGen primers, add the same volume of Illumina P5 and P7 primer mix (10 μM each of Illumina P5 and P7 primers). Follow with purification as in Steps 49–56

Timing

Steps 1–16, tissue collection and cell isolation: 4–5 h
Steps 17–21, microscopy staging using DAPI: 2 h
Steps 22–57, RNA-seq library preparation: 2 d
Steps 58–63, data sequencing and analysis: 4 d

Anticipated results

Step 41

After amplification by in vitro transcription, the user has the first opportunity to assess library quality. A high-quality library will have a small early peak, followed by a spread of density to the right that corresponds to the aRNA (Fig. 4a,b). If the libraries fail, there will only be a single small peak that can be seen, even in the absence of a template (Fig. 4c).

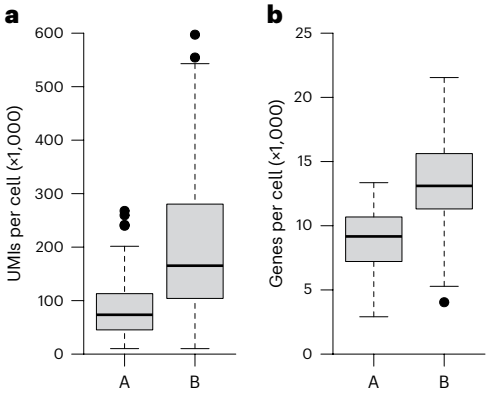

Fig. 5 | Read depth and gene diversity for scRNA-seq from isolated maize meiocytes. a, UMIs detected per cell from single isolated meiocytes. **b**, genes expressed per cell from single isolated meiocytes. Boxplots show the center line as the median, box limits as the upper and lower quartiles, whiskers as the 1.5× interquartile range and points as outliers; $N = 88$ in **a** and 139 in **b**. In each panel, the two boxplots were plotted by using data from ref. 3 (**a**) and ref. 4 (**b**). The first study used isolated protoplasts while the second used fixed cells following the protocol described here; both tissue dissociation methods produced libraries with high numbers of UMIs (>10,000 per cell) and resulted in similar gene expression patterns (see Supplementary Fig. 3 in ref. 4). To see expected UMIs per cell from other stages during pollen development, see Fig. 4a in ref. 4.

Step 57

CEL-seq DNA libraries should have a size distribution between 200 and 800 bp, with a peak around 300–400 bp (Fig. 4d). If density is observed below 200 bp, adapter dimers that can outcompete longer fragments when sequenced may be present, which indicates a need for additional size selection rounds (Fig. 4e). For library sequencing, the desired DNA concentration is >10 nM. If the size distribution is acceptable after the library quality check but the concentration is too low, the Illumina P5 and P7 primers can be used in additional rounds of PCR (see Troubleshooting).

Steps 62 and 63

Single maize meiotic cells and mature pollen grains generally produce a mean of 100,000–350,000 UMIs per cell or pollen grain (Fig. 5), while pollen precursors immediately after meiosis contain less RNA and produce an average of 10,000–40,000 UMIs per cell[4]. A mean of 8,000–15,000 genes are detected per cell at all stages. With this protocol, >70% of collected cells are expected to exceed 10,000 UMIs and 2,000 detected genes. When reanalyzing the data from ref. 3, ~35,000 UMIs are expected per cell, on average; this lower number is because (i) these libraries were from split-cell technical replicates and contain only the material for half a cell, and (ii) these libraries were made from protoplasts, which can have slightly lower UMIs per cell than fixed or fresh cells. Mapping rates for CEL-seq libraries are often low, ranging from 10% to 60%; the unmapped reads are predominantly ribosomal RNA, and we have not seen a high correlation between mapping rate and the quality of results. Mapping rates can be slightly improved by mapping to the genome rather than transcriptome, because the true 3′ UTR for some genes can extend beyond the annotated transcript region; see refs. 3,4 for example analyses with whole-genome mapping. High-quality libraries will meet the expected number of UMIs (>10,000) and expressed genes (>2,000) per cell.

Data availability

The example sequencing data analyzed in this protocol are available on the Gene Expression Omnibus under accession GSE121039. The data in Fig. 5 were reanalyzed from refs. 3,4 (Gene Expression Omnibus accessions GSE121039 and GSE175505). The 'data analysis' section can be followed by using example data from ref. 3, which can be accessed on the Sequence Read Archive under accessions SRR7989735 and SRR7989736.

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All authors contributed to the development and writing of this protocol.

Competing interests
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Key references using this protocol:
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