

# More cells, more doublets in sample-barcoded single-cell data

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## Abstract

Sample barcoding allows deconvolution of multiplets in multiplexed droplet-based single-cell RNA-sequencing experiments. However, this is only possible when each cell comes from a different sample. As the number of cells in a droplet increases, the probability of two or more cells coming from the same sample increases rapidly. We show that the number of these unresolvable multiplets is greater than previously estimated for the 10X Flex scRNA-seq protocol, and provide a formula for estimating the fraction of multiplets in a data set given a measured average droplet occupancy and number of unique samples in a pool. We also show that existing doublet detection tools should be applied to Flex data to identify these multiplets, and demonstrate that filtering out barcodes identified by these tools improves downstream analysis.

## Introduction

As single-cell RNA-sequencing (scRNA-seq) experiments grow in scale, performing separate captures for individual samples becomes prohibitively expensive. Pooling multiple samples in a single experiment both reduces cost and minimises batch effects, but introduces a new problem – how to associate individual cells with their sample of origin. A common method involves tagging the samples with either antibody or lipid-linked unique oligo barcodes prior to pooling. These “hashtag” oligos attach to either proteins or lipids on the cell surface and can be sequenced along with the gene expression, and various methods have been developed to identify the sample of origin from the expression counts of the hashtags [9]. However, hashtag-based multiplexing has several drawbacks. Firstly, tagging introduces an additional step into the sample preparation workflow, and may be more challenging for certain tissue types. Poor staining can cause many cells to be unidentifiable, even when the underlying RNA expression data is high quality [3]. Secondly, demultiplexing hashtag counts is a challenging computational problem, and the number of cells

that can be confidently assigned samples is dependent on the choice of demultiplexing software, especially for poor quality hashtagging [9].

In droplet-based scRNA-seq protocols, cells are encapsulated within droplets which contain oligo barcoded beads that hybridise to each RNA molecule in the cell. This allows for the allocation of reads to droplets, and, assuming each droplet contains either zero or one cell, each read can be associated with a single cell. If droplets contain two or more cells (called doublets, or more generally multiplets), the RNA expression from all the cells is pooled together in the expression counts matrix, creating artifacts which may be erroneously identified as novel or transitional cell types. Throughout this paper, we use the term “doublet” to refer to any droplet containing  $\geq 2$  cells. While multiplets can be identified based on their RNA expression profiles or using hashtags, they must be discarded from any downstream analysis [11]. Therefore, excessive numbers of multiplets are a major concern in droplet-based scRNA-seq experiments, particularly as the main approach for avoiding them is to reduce the capture throughput, thereby producing many more empty than cell-containing droplets, which in turn increases costs.

The Flex protocol from 10X Genomics addresses both of the above issues by using barcoded gene-specific probes that hybridise to individual RNA molecules in the cell. These barcodes can include an oligo that identifies a specific sample, with available kits containing 4 and 16 unique sample barcodes. The uniquely barcoded gene probes are added to each sample prior to pooling. Probe hybridisation is then followed by encapsulation within droplets containing a GEM (Gel beads-in-emulsion) bead similar to other 10X droplet-based protocols. After sequencing, each read then includes a barcode from both the sample of origin as well as the droplet, allowing immediate demultiplexing of pooled samples and resolution of multiplets from distinct samples within the same droplet. This removes the need for separate hashtag-staining and demultiplexing steps in the scRNA-seq workflow, and the resolution of multiplets allows for higher throughput in the library preparation stage. Consequently, according to the manufacturer, up to 128,000 cells from up to 16 samples can be run on a single lane of a Chromium X instrument [5].

However, while cells from different samples in the same droplet can be resolved with Flex, cells from the same sample remain unresolvable, and the number of these increases steeply with the occupancy of droplets. Here we estimate the number of multiplets expected in a Flex experiment and demonstrate that the number of multiplets found in real Flex data is significantly higher than initially expected. This is analogous to the “birthday paradox” in classical probability, where the probability of two people in a room sharing the same birthday increases faster than expected by intuition, as the number of people in the room increases.

## Results

### Flex data sets

We perform our analysis using two data sets acquired from ten 16-plex Flex runs. The first data set, the earlyAIR data set, comes from an ongoing study to create a single-cell atlas of the paediatric airway, and comprises nine 16-plex batches from five tissue types, comprising both solid and liquid tissues from healthy donors. Some donors provided multiple tissues, and the batches contain between 25,486 and 139,926 cells after pre-processing (see **Methods**). The second data set, the PBMC data set, comprises 16 replicates of peripheral blood mononuclear cells taken from a healthy donor and is publicly available from 10X Genomics (see **Data availability**). The PBMC data set contains 135,785 cells after pre-processing and filtering.

### Testing doublet finders with known multiplets

Tests of doublet-finding methods have to date relied upon a ground truth determined from alternative experiments, such as genotype information [11], hashtag-based demultiplexing [4], DNA barcoding [15], or synthetic data [12].

Flex provides a novel means of testing doublet finding directly from sequencing. In a Flex experiment, many droplets contain multiple cells and cell calling is performed separately for each sample barcode. This means the initial step in a standard analysis is to separate reads based on their combined droplet and sample barcode and then call cells or empty “droplets” based on the data separated by samples. A droplet/sample combination may look empty even when there is a cell from a different sample in the same droplet. The resulting counts matrix has cells labelled by both the droplet barcode and sample barcode. Aggregating the counts for each droplet across the sample barcodes creates a droplet-level counts matrix analogous to 3’ scRNA-seq experiments. Since the number of cells (from unique samples) called by Cell Ranger in each droplet is known, the droplet-level counts matrix provides a ground truth data set for testing doublet finders: droplets with only one cell are singlets and droplets with

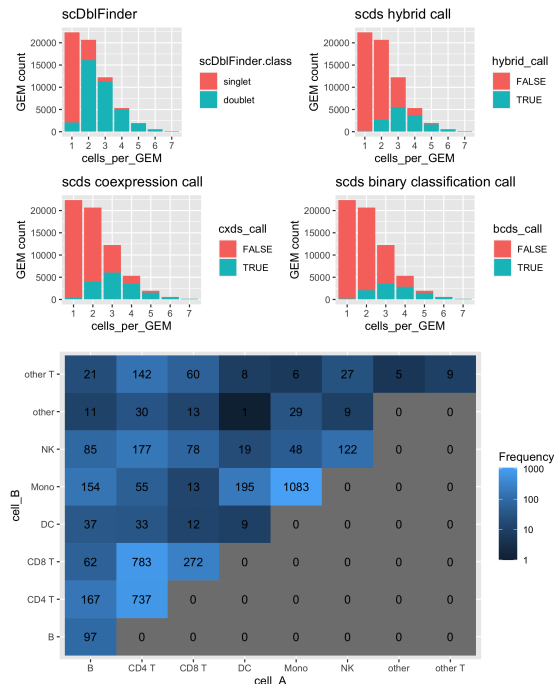


Fig. 1: a-d) Performance of doublet-finders on droplets with occupancy determined by Cell Ranger calls. Each bar represents the identified number of cells in a droplet based on the sample deconvolution. The blue portion represents the number of detected doublets from the doublet detection tool. Ideally, a tool will only detect singlets where there is one cell per GEM and doublets when there are more than one cell. Annotation of cell types in droplets containing two cells misclassified as singlets by scDbtFinder. The majority (>50%) of cells misclassified as singlets are homotypic doublets.

two or more cells are doublets. Note that we do not intend this section as a comprehensive benchmark of doublet-finding methods, which may be found in [11]. Instead, we use two of the best-performing packages with low computational overhead recommended in [11]: scds [2] and scDbtFinder [6] and see which performs best on our Flex data sets. scds has two methods for doublet detection, one based on co-expression and another based on binary classification, as well as a hybrid classifier that combines both methods. We use all approaches and some details of how each method works and the parameters chosen can be found in the Methods section.

Figure 1 shows the performance of scDbtFinder and the two scds methods as well as the hybrid classifier on the droplet-aggregated 10X PBMC data set. For this data, scDbtFinder (Figure 1a) clearly outperforms the scds methods (Figure 1 b-d), with 90% of the multiply-occupied droplets called as doublets compared to only 25%-40%. Doublet-finding methods are known to perform poorly on droplets containing multiple cells from the same type (homotypic doublets), as these are unlikely to show discrepant transcription profiles [13, 11]. To examine whether this is the case for the known multiplets in the 10X PBMC data set misclassified by scDbtFinder, we first annotate the cells using the reference mapping package Azimuth and its included PBMC reference data set [8]. We show the annotated cell types of both cells in the droplets containing two cells that are called as singlets by scDbtFinder in Figure 1. The majority of the misclassified doublets are homotypic doublets (50.5%) or have more

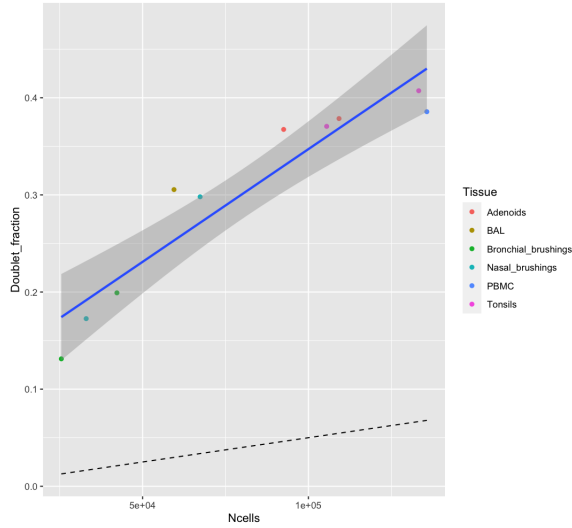


Fig. 2: Doublet fraction (from scDblFinder) vs number of cells called for 10 16-plex Flex data sets. The blue line indicates a linear fit to the data. The black dashed line is the estimated rate from the Flex user guide.

closely related transcriptional profiles such as CD8+ T cells with CD4+ T cells (X%). scDblFinder calls 9.2% of the CellRanger singlets as doublets, compared to ~2% for scds. In the following sections, we examine doublets in more depth and show that the rate of unresolved doublets predicted by our mathematical model is quite close to the value from scDblFinder.

### Measuring multiplet rates in Flex data

Having identified scDblFinder [6] as our preferred doublet-finding method for this study, we run it on all of our data sets. Figure 2 shows the fraction of doublets called by scDblFinder on each Flex run as a function of the number of cells. Also shown in Figure 2 is the predicted doublet fraction from the documentation for the Flex protocol [5] of 0.8% per 1000 cells per sample. A clear linear trend is observed in the results from scDblFinder, with a best fit multiplet rate of 2.3% per 1000 cells per sample, or  $\approx 4$  times more than the estimate from the documentation.

### Estimating the resolvable cell fraction

Why is the multiplet rate predicted by doublet finders so much greater than the rate estimated by the manufacturer? Recall that the quoted rate of unresolvable multiplets in Flex comes from dividing the rate for the 3' protocol by the number of samples. This makes sense if each Flex sample is processed separately at the same concentration as an equivalent 3' sample, but the recommended practice is to pool all samples together after probe hybridisation, so that the concentration of cells is up to 16 times greater than a 3' experiment sequencing the same number of cells per sample. 3' experiments are designed to avoid multiplets, so that very few droplets contain more than one cell. In Flex experiments the ability to resolve cells from different samples within the same droplet means that total cell concentrations can be much larger, and droplets containing multiple cells are common (cf. Figure 1). In this section, we perform some calculations showing that increasing the occupancy of droplets leads to a higher number of unresolved cells than previously thought.

Consider an ensemble of droplets containing  $k$  cells, drawn from a pool containing  $n$  samples with equal proportions of cells from all samples. The number of possible configurations of cells within the droplets is the number

of combinations of length  $k$  drawn from  $n$  with repetition allowed, i.e.

$$M_k(n) = \frac{(n+k-1)!}{(n-1)!k!}, \quad (1)$$

where  $!$  denotes the factorial function. Assuming each of these combinations is equally probable, we aim to determine what fraction  $F(n, k)$  of cells in droplets containing  $k$  cells are “resolvable”, which we define as being the only cell from their sample of origin. In general,

$$F(n, k) = \frac{\sum_c f_c}{M_k} \quad (2)$$

where the subscript  $c$  denotes a single configuration of cells within a droplet, and  $f_c$  is the resolvable fraction of that configuration. Solving (2) therefore requires determining  $f_c$  for each combination.

We define the observed occupancy of a droplet as  $l$ ,  $l \leq k$ , as the number of unique samples in a droplet. Droplets with  $k = 1$  are all resolvable. For droplets with  $k \geq 2$ , if all cells in the droplet are from the same sample, i.e.  $l = 1$ , no cells are resolvable, while if each cell comes from a unique sample, i.e.  $l = k$ , all cells are resolvable. In general, we can use the concept of integer partition to determine the resolvable fraction of a given combination of cells. A partition is a way of writing an integer  $k$  as a sum of integer “parts”, e.g. the partitions of 5 are (5), (4 + 1), (3 + 2), (3 + 1 + 1), (2 + 2 + 1), (2 + 1 + 1 + 1), (1 + 1 + 1 + 1 + 1) [1]. In this picture, each part of the partition is a unique sample, the number of parts is the observed occupancy  $l$ , and the number of 1s in the partition is the number of resolvable cells, e.g., the partition (2 + 2 + 1) represents a droplet with  $k = 5$  consisting of 2 cells from sample A, 2 cells from sample B and 1 cell from sample C. We can define the fraction of resolvable cells in a droplet  $f$  in two ways: first, the number of resolvable cells from unique samples in the droplet divided by the total number of cells in the droplet, i.e. the number of 1s in the partition divided by  $k$ ; second, the number of resolvable cells divided by the number of unique samples in the droplet, i.e. the number of 1s in the partition divided by  $l$ . Since we are interested in comparing to experimental data, we use the second definition. For the partitions of 5 written above, this gives  $f = 0, 0.5, 2/3, 1/3, 0.75, 1$ .

Since any configuration of cells within a droplet can be represented as a partition, and the resolvable fraction of each partition is easily calculable [1], all that we need to solve (2) is the proportion each partition contributes to the total number of combinations  $M_k$  [equation (1)]. We denote each of the partitions of  $k$   $P_i(k)$ . Define  $m_j(P_i)$  the number of occurrences of  $j \in \{1, \dots, k\}$  in  $P_i$ . The observed occupancy,  $l(P_i)$  is the length of the partition and the resolvable fraction of  $P_i$ ,  $f(P_i) = m_1(P_i)/l$ . To determine the number of combinations corresponding to  $P_i$ ,  $C_i(n, k)$ , consider the first non-zero  $m_j$ . There are  $\binom{n}{m_j}$  possible combinations of samples for these cells, where

$$\binom{n}{m_j} = \frac{n!}{(n-m_j)!m_j!} \quad (3)$$

is the binomial coefficient. For the next non-zero coefficient,  $m_h$ , there will be  $\binom{n-m_j}{m_h}$  ways of choosing those samples, and in general we expect

$$C_i(n, k) = \prod_{j=1}^k \binom{n - \sum_{h < j} m_h}{m_j} \quad (4)$$

As a check on (4), we find that

$$\sum_i C_i(n, k) = M_k(n) \quad (5)$$

as required. The resolvable fraction of cells in droplets containing  $k$  cells is therefore

$$F(n, k) = \sum_i \frac{f(P_i) C_i}{M_k(n)}. \quad (6)$$

We show  $F$  versus  $k$  for  $n = 4$  and  $n = 16$  in Figure 3. Figure 3 shows

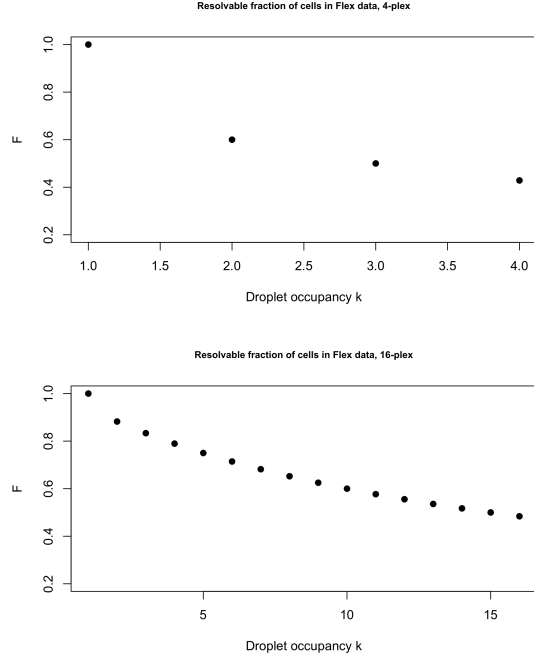


Fig. 3: Fraction of resolvable cells  $F(n, k)$  vs occupancy  $k$  for  $n = 4$  (top) and  $n = 16$  (bottom).

that  $F_r$  decreases steeply with  $k$ , with  $F(n = 4) < 0.5$ , for  $k > 2$  and  $F_r(n = 16) < 0.75$  for  $k > 5$ .

To determine the size of the effect in real data, we need to combine equation (6) with the distribution of droplet occupancy  $k$ . A good model for the distribution of observed occupancy  $l$  in Flex data is the zero-truncated Poisson distribution (see **Supplementary Materials**)

$$p(l) = \frac{\lambda^l}{l!(e^\lambda - 1)}, \quad (7)$$

where  $\lambda$  is a shape parameter related to the mean droplet occupancy  $\langle l \rangle$  through

$$\langle l \rangle = \frac{\lambda}{1 - e^{-\lambda}}. \quad (8)$$

We now make a simplifying assumption that the distribution of the true occupancy  $k$  can be approximated by the distribution of the observed occupancy  $l$ , i.e.

$$p(k) \approx p(l). \quad (9)$$

We make this assumption in order to simplify what follows, and because determination of a relationship between  $k$  and  $l$  is a non-trivial task requiring a more robust ground-truth data set for known doublets in Flex data than is currently available. The number of cells in droplets with observed occupancy  $k$  is then

$$N_{\text{cells}}(k) = N_{\text{droplets}} k p(k). \quad (10)$$

Combining equations (6) – (10), we get the fraction of multiplets in an experiment

$$M_f(n) = 1 - \sum_k \frac{k p(k) F_r(n, k)}{\langle l \rangle}. \quad (11)$$

We show the expected doublet fraction in data sets for  $n = 4$  and  $n = 16$  as a function of mean observed droplet occupancy  $\langle l \rangle$  in Figure 4. Figure 4

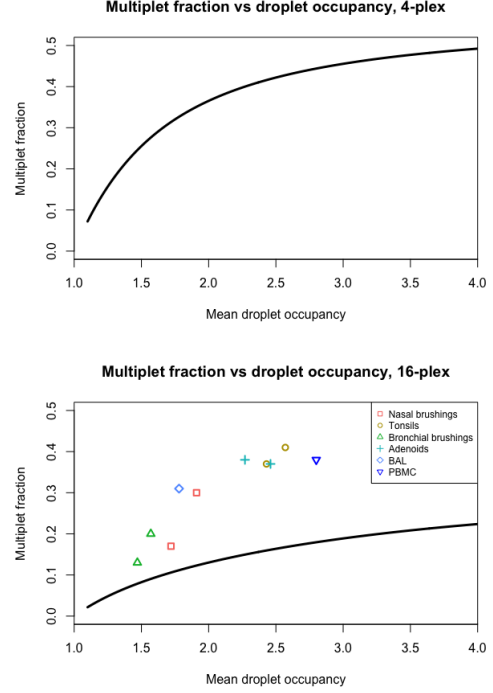


Fig. 4: Calculated fraction of multiplets in Flex data vs mean droplet occupancy for  $n = 4$  (top) and  $n = 16$  (bottom). Coloured points indicate doublet fraction determined from scDblFinder and observed mean droplet occupancy from earlyAIR and PBMC data sets.

also includes the doublet fraction from scDblFinder for the earlyAIR and PBMC data sets in the 16-plex case [6]. Figure 4 shows that the predicted multiplet fraction from equation (11) still underestimates the doublet fraction in real-world data, though by significantly less than the prediction from the Flex User Guide [5]. We propose two potential reasons for this discrepancy. The first is the assumption that the true occupancy distribution is the same as the observed distribution, equation 9. The presence of unresolvable cells implies that the true occupancy is always greater than that observed, see **Supplementary materials**. Properly accounting for this effect would shift the data points in Figure 4 to the right, bringing them closer to the predicted curve. Another reason is the assumption that all samples have equal numbers of cells. In any experiment, some samples contain more cells than others. We expect that this increases the likelihood of same-sample multiplets, though exactly how is beyond the scope of this work.

Even with the limiting assumptions of our model, however, the fraction of unresolved multiplets in an experiment with a mean droplet occupancy of 2.5 (a typical value for an experiment with the maximum recommended loading of 128,000 cells) is 0.17, almost 3 times greater than the value of 0.064 estimated by 10X. These results demonstrate the necessity of using a doublet detection method to remove doublets from Flex data.

Finally, in Figure 1b we observe that scDblFinder calls 2060 of the droplets in the PBMC data set identified as singlets by CellRanger as doublets. We can estimate the number of observed singlets that are actually doublets using equation (6) and the observed distribution of droplet occupancies. From this, we estimate that  $\sim 2700$  droplets with occupancy  $k > 1$  will appear as singlets in this data set. Most of these will have  $k = 2$  (2 cells, 2400 droplets), but some droplets can contain more cells



Fig. 5: a) Number of clusters as a function of cluster resolution for PBMC data set before (red crosses) and after (blue circles) removal of doublets identified by scDbtFinder. b) Number of cells in each cluster in the PBMC data set at resolution 0.4, coloured by fraction of doublets. c) same as b) but with proportions instead of total counts. d) UMAP showing cells in the PBMC data set with labels from Azimuth. e) Same as d) but showing only cells from clusters with  $\geq 75\%$  doublets. f) UMAP with Azimuth labels computed after removing doublets.

from the same sample (**Supplementary figure**). The number predicted by the model is similar to the value of 2,060 from scDbtFinder, with the discrepancy likely due to the difficulty of scDbtFinder in identifying homotypic doublets, shown in Figures 1b and ??.

### Doublet-finding identifies spurious clusters

In order to demonstrate the effect of doublets on downstream analysis, we perform unsupervised clustering on the PBMC data set at a range of resolutions from 0.1 to 1.0 using the FindMarkers function with the SLM algorithm in Seurat. We use two versions of the data: one with all the cells that pass our initial QC filtering, and another and another after removing all of the doublets identified by scDbtFinder. At every resolution, removing the doublets reduces the number of clusters (Figure 5a), indicating that doublets introduce structure in the data.

We next look at the doublet composition of the identified cluster at resolution 0.4, (Figures 5b and c). We identify 11 clusters that contain more than twice the average doublet fraction of the entire data set. We show the position of all cells in the data set prior to doublet removal, labelled by their Azimuth annotations, on a Uniform Manifold Approximation and Projection (UMAP) plot in Figure 5d. Figure 5e shows only the cells in the 11 clusters identified as being composed of mostly doublets. Figures 5d and e show that these doublet-dominated clusters appear in the regions

of the UMAP that bridge multiple cell types, such as CD14 and CD16 monocytes, or T cells and NK cells, lending further evidence for their identity as doublets.

Lastly, Figure 5f shows the UMAP with Azimuth labels after removing the doublets. In Figure 5f, certain cell populations, such as T and NK cells, CD4 T cells and CD8 T cells, and CD14 and CD16 Monocytes, are better separated in more distinct clusters than in Figure 5d.

## Discussion

Sample barcoding, as implemented in the 10X Flex protocol, offers a solution to two significant issues in droplet-based scRNA-seq experiments: robust identification of sample-of-origin in multiplexed cell pools, and deconvolution of multiple cells from different samples in the same droplet. However, we show that unresolvable doublets, consisting of multiple cells from the same sample, remain a significant issue in Flex data sets, with scDbtFinder classifying several times more cells as doublets than predicted by the manufacturer.

We verify that the predictions of scDbtFinder are accurate by aggregating the droplet counts across the sample barcodes, a novel approach that will prove useful in future benchmarking studies of doublet-finding algorithms. We then propose a mathematical model for predicting the number of same-sample doublets in any droplet-based single-cell protocol with sample tagging, and find that the predictions of our model are much closer to the observed doublet rates in Flex experiments than the estimates from the user documentation. We demonstrate that doublet removal improves downstream analysis by eliminating spurious cell clusters consisting primarily of doublets.

Despite the greater-than-expected number of doublets in Flex data, we find that in general the Flex protocol provides similar data quality to previous 3' scRNA-seq protocols, and existing tools and workflows, such as ambient RNA removal and cell annotation, work without issue.

## Methods

### Single-cell data pre-processing strategy

The starting point for all data sets discussed in this paper is a counts matrix consisting of cells called by Cell Ranger. This data is pre-processed in the following order prior to running doublet-finding tools.

- Removal of unexpressed genes.
- Removal of ambient background contamination using decontX [14].
- Removal of cells with fewer than 250 reads.
- Removal of cells with high mitochondrial read percentages, using the isOutlier function in the DropletUtils package [7, 10].
- Doublet calling with scDbtFinder [6]

### Data availability

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