

1 Estimating the frequency of multiplets in 2 single-cell RNA sequencing from 3 cell-mixing experiments

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9 ABSTRACT

10 In single-cell RNA-sequencing, it is important to know the frequency at which the sequenced transcriptomes actually derive from multiple cells. A common method to estimate this multiplet frequency is to mix
11 two different types of cells (e.g., human and mouse), and then determine how often the transcriptomes
12 contain transcripts from both cell types. When the two cell types are mixed in equal proportion, the
13 calculation of the multiplet frequency from the frequency of mixed transcriptomes is straightforward.
14 But surprisingly, there are no published descriptions of how to calculate the multiplet frequency in the
15 general case when the cell types are mixed unequally. Here I derive equations to analytically calculate
16 the multiplet frequency from the numbers of observed pure and mixed transcriptomes when two cell types
17 are mixed in arbitrary proportions, under the assumption that the loading of cells into droplets or wells is
18 Poisson.
19

20 INTRODUCTION

21 Many methods for single-cell RNA sequencing involve partitioning cells into barcoded droplets (Klein
22 et al., 2015; Macosko et al., 2015; Zheng et al., 2017), wells (Gierahn et al., 2017), or combinations of
23 wells (Cao et al., 2017). As long as the number of possible partitions exceeds the number of cells, then
24 most partitions will contain at most one cell. However, some fraction of the non-empty partitions will
25 contain multiple cells, and estimating this *multiplet frequency* is an important aspect of experimental
26 quality control.

27 The most common method to determine the multiplet frequency is to ~~use a~~-mix two types of cells
28 (e.g., human and mouse). During the analysis of the sequencing results, each non-empty partition can
29 be identified as containing transcripts from one or both of the two cell types. Partitions that contain a
30 substantial number of transcripts from both cell types must be multiplets. If the two cell types are mixed
31 equally and the average number of cells per partition is low (so that most multiplets are doublets), then the
32 multiplet frequency can be estimated as simply twice the fraction of non-empty partitions that contain a
33 mix of cell types. The logic is that all the multiplets are doublets, and only half the doublets will have cells
34 of both types (the others will have two cells of the same type). This approach has been used to estimate
35 the multiplet frequency during the prototyping of most single-cell RNA sequencing methods (Klein et al.,
36 2015; Macosko et al., 2015; Zheng et al., 2017; Gierahn et al., 2017; Cao et al., 2017).

37 However, in some cases the two cell types may be mixed in unequal proportions. Unequal mixing
38 could arise simply from error during cell counting, ~~but it can also be a desirable or it could be an~~
39 intentional aspect of experimental design (Rosenberg et al., 2018). For instance, if the researcher is
40 actually interested in the human cells and simply wants to include an internal control to estimate the
41 multiplet frequency during each new experiment, then (s)he may want to add fewer mouse cells so
42 that most of the resulting data is for the human cells. In addition, when analyzing naturally occurring
43 mixtures of cells of multiple types, the different cell types will usually be present in unequal proportions.
44 But when the cells are mixed unequally, it is no longer valid to estimate the multiplet frequency as simply
45 twice the fraction of non-empty partitions that contain a mix of both cell types. Surprisingly, I could

find no published descriptions of how to calculate the multiplet frequency from unequal mixes of two cell types. Here I remedy this gap in the literature by deriving the equations to compute the multiplet frequency when the cells are mixed in arbitrary proportions under the assumption that the number of cells per partition is Poisson distributed. This Poisson assumption is accurate when cells are loaded randomly and independently into partitions.

METHODS

The LaTeX source for this paper, the Jupyter ~~notebook that implements~~ notebooks that implement the calculations, and all materials associated with the writing and review of the paper are publicly available in a GitHub repository at https://github.com/jbloomlab/multiplet_freq. The Jupyter ~~notebook is~~ notebooks are also available in Supplemental file 1 ~~; and an HTML rendering of that notebook is and~~ 3, and HTML renderings of the notebooks are in Supplemental file 2 and 4.

RESULTS

Derivation of multiplet frequency from observed numbers of pure and mixed-cell droplets

Consider the case in which cells of two types (e.g., human and mouse) are distributed into individual barcoded droplets, although the same logic applies if the cells are distributed into barcoded wells or combinations of wells. Assume the sequencing data have been analyzed so that each non-empty droplet can be classified as containing at least one cell of type 1, at least one cell of type 2, or cells of both types. I will refer to the number of droplets in each of these three groupings as N_1 , N_2 , and $N_{1,2}$, respectively. For instance, the 10X `cellranger` pipeline (version 2.1.1) returns these numbers as the “Estimated Number of Cell Partitions.”

The only assumption of the derivation is that the number of cells per droplet is Poisson distributed. Let μ_1 be the average number of cells of type 1 per droplet, and μ_2 be the average number of cells of type 2 per droplet. The average number of cells of any type per droplet is then $\mu_1 + \mu_2$. So the probability that a droplet contains at least one cell of any type is

$$\begin{aligned}\Pr(c \geq 1) &= 1 - \Pr(c = 0) \\ &= 1 - e^{-\mu_1 - \mu_2}.\end{aligned}\tag{1}$$

Likewise, the probability that a droplet contains multiple cells of any type (e.g., a multiplet) is

$$\begin{aligned}\Pr(c \geq 2) &= 1 - \Pr(c = 0) - \Pr(c = 1) \\ &= 1 - e^{-\mu_1 - \mu_2} - (\mu_1 + \mu_2)e^{-\mu_1 - \mu_2}.\end{aligned}\tag{2}$$

The multiplet frequency M is simply the probability that a droplet with at least one cell actually contains multiple cells, which is

$$\begin{aligned}M &= \frac{\Pr(c \geq 2)}{\Pr(c \geq 1)} \\ &= 1 - \frac{(\mu_1 + \mu_2)e^{-\mu_1 - \mu_2}}{1 - e^{-\mu_1 - \mu_2}}.\end{aligned}\tag{3}$$

However, evaluating this expression for M requires the values of μ_1 and μ_2 .

We can write down equations for μ_1 and μ_2 by again using the fact that the number of cells per droplet is Poisson distributed. Specifically, if N is the total number of droplets (empty and non-empty), then the expected number of droplets that have at least one cell of type 1 is $N \times \Pr(c_1 \geq 1) = N(1 - e^{-\mu_1})$. The observed number of droplets with at least one cell of type 1 is N_1 , so setting the observed number equal to the expected number gives us an equation for μ_1 ,

$$N_1 = N(1 - e^{-\mu_1}).\tag{4}$$

This equation is easily solved for μ_1 to yield

$$\mu_1 = -\ln\left(\frac{N - N_1}{N}\right),\tag{5}$$

and likewise for μ_2 ,

$$\mu_2 = -\ln\left(\frac{N - N_2}{N}\right). \quad (6)$$

Equations 5 and 6 give us a way to determine the values (μ_1 and μ_2) needed to calculate the multiplet frequency (Equation 3) in terms of the experimental observables N_1 and N_2 . Unfortunately, these two equations also require knowledge of the total (empty and non-empty) number of droplets N , which is not directly observable from the sequencing data.

However, we can take advantage of another relationship to calculate N . The fraction of all (empty and non-empty) droplets that contain cells of both types is $\frac{N_{1,2}}{N}$, and this fraction is simply the product of the probability that a droplet contains at least one cell of type 1 with the probability that a droplet contains at least one cell of type 2, which in mathematical terms can be stated as $\Pr(c_1 \geq 1 \wedge c_2 \geq 1) = \Pr(c_1 \geq 1) \times \Pr(c_2 \geq 1)$. Therefore,

$$\frac{N_{1,2}}{N} = \frac{N_1}{N} \times \frac{N_2}{N}. \quad (7)$$

This equation can be solved to give

$$N = \frac{N_1 N_2}{N_{1,2}}, \quad (8)$$

which can be completely evaluated in terms of the experimental observables. Equations 5, 6, and 8 can be used to calculate μ_1 and μ_2 in terms of the experimental observables, and those results used to calculate the multiplet frequency via Equation 3. This provides an analytic solution for the multiplet frequency in terms of the three experimental observables.

Implementation and example calculations

A simple function to perform the calculations described in the previous subsection is implemented in Python [and in the Jupyter notebook found at `https://github.com/jbloomlab/multiplet_freq/blob/master/calcmultiplet.ipynb`](https://github.com/jbloomlab/multiplet_freq/blob/master/calcmultiplet.ipynb), and in R in the Jupyter notebook found at https://github.com/jbloomlab/multiplet_freq/blob/master/calcmultiplet_R.ipynb (see also Supplemental files [1](#) and [2, 3, and 4](#)). To illustrate the calculations, I used this function to calculate the multiplet frequency for hypothetical data.

First, consider hypothetical data in which the two types of cells are mixed in equal proportions. Prior papers have approximated the multiplet frequency from such experiments as simply twice the fraction of non-empty droplets that contain cells of both types (Klein et al., 2015; Macosko et al., 2015; Zheng et al., 2017; Cao et al., 2017), which is $\frac{N_{1,2}}{N_1 + N_2 - N_{1,2}}$ in the notation defined in the previous subsection. Table 1 shows that the exact equation derived in the previous subsection gives very similar results to this approximate method as long as the multiplet frequency is low. When the multiplet frequency becomes high, the approximate method starts to overestimate the true multiplet frequency, since it fails to account for the fact that some multiplets will contain more than two cells.

Next, consider hypothetical data in which the two types of cells are mixed in unequal proportions. Table 2 shows the multiplet frequencies for several such experiments. An interesting aspect of the results is that at high multiplet frequencies and very unequal cell proportions, the multiplet frequency is substantially *lower* than the fraction of droplets containing the rarer cell type that contain a mix of both cell types. The reason is that multiplets (particularly higher-order ones) become more and more likely to contain at least one cell of the rarer type relative to droplets that contain only one cell. For instance, in the final experiment in Table 2, two thirds of the droplets containing mouse cells have a mix of both cell types, yet less than half the non-empty droplets are multiplets (the multiplet frequency is 0.459). This somewhat non-intuitive [results-result](#) illustrates the importance of using the correct mathematical relationship to calculate the multiplet frequency when cell types are mixed unequally.

CONCLUSIONS

I have described how to calculate the multiplet frequency in single-cell RNA sequencing experiments in which two cell types are mixed in arbitrary proportions. It is important to note that this calculation

requires that the sequencing data have already been analyzed to determine whether each partition contains a non-negligible number of transcripts from each cell type, but many common analysis programs (such as the 10X cellranger pipeline) already do this. ~~It is also important to note that the~~

The calculation also assumes that the number of cells per droplets follows a Poisson distribution. While many single-cell RNA sequencing methods are designed to partition cells in a way that concords with this assumption (Klein et al., 2015; Macosko et al., 2015; Zheng et al., 2017; Gierahn et al., 2017; Cao et al., 2018), it is possible that cell clumping or other factors could bias certain partitions to contain more cells than expected under a Poisson distribution. In such a scenario, the calculations in this paper would overestimate the true multiplet frequency if the clumping is equally likely across cell types, but could underestimate the true multiplet frequency if intra-cell-type clumping is more likely than inter-cell-type clumping.

Finally, the approach in this paper only calculates the multiplet frequency—it does *not* actually identify the multiplets so that they can be removed from downstream analyses. For that purpose, other more sophisticated approaches have been developed (Ilicic et al., 2016; Stoeckius et al., 2017; Kang et al., 2018) (Ilicic et al., 2016; Stoeckius et al., 2017; Kang et al., 2018; Wolock et al., 2018; DePasquale et al., 2018). Nonetheless, simply calculating the multiplet frequency from the data returned by standard pipelines such as the 10X cellranger is important for many purposes, and the results here enable that to be done regardless of the proportions at which the cell types are mixed.

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experiment	human droplets	mouse droplets	nonempty droplets	human and mouse droplets	multiplet freq	twice cross celltype freq
1	2005	2005	4000	10	0.005	0.005
2	2050	2050	4000	100	0.049	0.050
3	2500	2500	4000	1000	0.425	0.500

Table 1. Multiplet frequencies for three hypothetical experiments in which human and mouse cells are mixed equally. The multiplet frequencies calculated using the exact method described here (column *multiplet freq*) are very similar to those obtained simply by multiplying by two the fraction of non-empty droplets that contain cells of both types (column *twice cross celltype freq*). However, the two methods are slightly different at higher multiplet frequencies, since the latter method fails to account for multiplets that have more than two cells.

experiment	human droplets	mouse droplets	nonempty droplets	human and mouse droplets	multiplet freq
1	2050	2050	4000	100	0.049
2	3050	1050	4000	100	0.065
3	3550	550	4000	100	0.110
4	3850	250	4000	100	0.245
5	3950	150	4000	100	0.459

Table 2. Multiplet frequencies for five hypothetical experiments in which human and mouse cells are mixed unequally.

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Supplemental file 1. A Jupyter notebook that implements the calculations in Python~~and R-functions~~, and does the calculations for the examples shown in the tables in this paper.

Supplemental file 2. This file contains an HTML rendering of the Jupyter notebook in Supplemental file 1.

Supplemental file 3. [A Jupyter notebook that implements the calculations in R, and does the calculations for the examples shown in the tables in this paper.](#)

Supplemental file 4. [This file contains an HTML rendering of the Jupyter notebook in Supplemental file 3.](#)