Supp Figure 1. HipSci data sparsity, replicates, and genotyping.

Supp Figure 2. Maternal/Fetal decidua1 and placenta2.

Supp Figure 3. Plasmodium clustering.

Demonstration on Demuxlet paper data

In order to demonstrate souporcell on an external and widely used benchmark dataset, we downloaded the three overlapping mixtures from the demuxlet paper[6]. Sample A contains a mixture of four donors' PBMCs, Sample B contains a mixture of four different donors' PBMCs, and Sample C contains a mixture of all 8 donors' PBMCs. We synthetically combined this data into a single dataset and clustered with souporcell. Supp Fig 4a shows that the resulting clusters either contain cells from Sample A or Sample B, but not both as is expected from this experimental setup. We also show that the first cluster of the doublet assignments are also largely consistent with this experimental design (Supp Fig 4b).

Supp Figure 4. Demuxlet data

Deconvolution of overlapping mixtures

To enable identification of which cluster is which individual using the overlapping mixture experimental design outlined in Table 1, we provide a tool shared_samples.py which takes as input two souporcell output directories and the number of samples which are shared. It compares the sum of squared differences of the allele fraction of confident (>95% confident genotype call in all clusters) shared variant calls between clusters in the two experiments and outputs the best matches for the number of shared samples. We tested this using multiple different runs of souporcell on the synthetic mixture of 5 HipSci cell lines with 6% doublets and 5% ambient RNA and gave both as input to the shared_samples.py tool and it correctly assigned the clusters in one run to the clusters in the second experiment which corresponded to the same samples. We also ran souporcell on the three demuxlet datasets separately and ran the shared_samples.py tool on Sample A vs Sample C and Sample B vs Sample C and it confidently identified the non-overlapping clusters in Sample C which correspond to A and B.

Demonstration on 21 donor sample

We demonstrate that souporcell is capable of demultiplexing many donors by creating a synthetic mixture of 21 different individuals, which given the current recommendations from 10x on cells per run would be a high-end number of donors to multiplex. To generate this 21-donor mix, we used the 5 HipSci samples described in Fig 2 and added to them 16 PBMC samples obtained from the Human Cell Atlas Census of Immune Cells[25]. From each dataset we randomly selected 1000 cells with at least 4000 UMIs and simulated 10% doublets and 2.5% ambient RNA by altering the cell barcodes, as described above. We clustered these

with souporcell and the software correctly identifies 1690 of the 2100 synthetic doublets. A further 69 cells were unassigned, and in total we have an ARI of 0.95. Excluding all doublets the ARI is 0.98. We find a total of 134/16800 singletons misassigned where 129 of them are CB8 cells assigned to the CB3 cluster. We show later that this is likely because the CB8 sample is contaminated by another (non CB3) donor. Supp Figure 5 shows the UMAP projection of the normalized cluster log likelihood matrix. It is clear that souporcell is able to handle at least 21 distinct donors and accurately assign cluster identities to the majority of cells.

Supp Figure 5. 21 donor synthetic mixture.

Because this error type accounted for >95% of singleton errors, we suspected this may be due to contamination. We repeated this experiment with several of the replicates of the CB8 donor and found consistent results. We then made a synthetic mixture of CB3 and CB8 in order to determine if this was due to the large number of donors and it was not. We still found that roughly 20% of CB8 cells would cluster with CB3, but if given 3 clusters, all of those cells formed their own cluster. This made us suspect that the CB8 sample was contaminated with cells from a different (non CB3) donor. We created an elbow plot and a PCA of the normalized cell x cluster log likelihood matrix, and both support this hypothesis (Supp Fig 6). In communications with the Broad Institute, they confirm that the genomics platform reported 19% contamination with this sample, perhaps from maternal blood as this was a cord blood sample.

Supp Figure 6. Contamination of CB8 samples

Downsampling experiments for cells and UMIs

In order to explore the regime for which it is still possible to accurately demultiplex mixed samples, we used our synthetically mixed 5 HipSci samples and downsampled UMIs (Supp Fig 6a) and cell (Supp Fig 6b) and report the ARI versus the ground truth. We find that while overall clustering remains good, cell assignment accuracy decreases below 800 median UMI per cell and that accuracy remains high down to an average of 40 cells per cluster (Supp Fig 6b).

Supp Figure 7. UMI and Cell downsampling.

Software versions

Initial data analysis was done by cellranger v2.1.1 as input to souporcell, demuxlet, vireo, and scSplit

Souporcell

https://github.com/wheaton5/souporcell

We provide a singularity container build encapsulating all requirements for souporcell as well as a singularity

definition file to recreate this container. The following are the software versions for all software excluding software required to build the system.
Freebayes - v1.3.1-17-gaa2ace8
Pyvcf - 0.6.7
Pysam - 0.15.3
Numpy - 1.17.0
Scipy - 1.3.0
Tensorflow - 1.14.0
Pystan - 2.17.1.0
Pyfasta - 0.5.2
Htslib - 1.9
Samtools - 1.9
Bcftools - 1.9
Vartrix - 1.1.3
Minimap2 - 2.7-r654
Bedtools - 2.28.0

Demuxlet
https://github.com/statgen/demuxlet
git hash 85dca0a4d648d18e6b240a2298672394fe10c6e6

Vireo
Cardelino R package version 0.3.8
cellSNP (https://github.com/huangyh09/cellSNP version 0.1.6)

scSplit
Freebayes - v1.3.1-17-gaa2ace8
https://github.com/jon-xu/scSplit git commit hash 52face6a4c1b291651bdf9b56328d168c7cb1fa6

Supp Table 1: Sample metrics.

Sample	Cells	Median UMI/cell	NOTES
Mixture1	4925	25155	HipSci
Mixture2	4832	24913	HipSci
Mixture3	5144	24807	HipSci
euts	4859	25088	HipSci
nufh	6781	17254	HipSci
babz	12299	11343	HipSci
oaqd	7107	18315	HipSci
ieki	6586	21969	HipSci
SyntheticMixture	7073	18793	synthetic HipSci mixture (5% ambient RNA, 6% doublets)
Placenta1	3835	18415	Maternal Fetal
Placenta2	3968	18046	Maternal Fetal
Decidua1	2119	23075	Maternal Fetal

Plasmodium1	2608	995	*Plasmodium falciparum*
Plasmodium2	1893	762	*Plasmodium falciparum*
Plasmodium3	2293	1126	*Plasmodium falciparum*

Number of cells and median UMI/cell for each sample including one representative synthetic mixture.

Supp Table 2: Hipsci clustering.

	method	mixture1	mixture2	mixture3	synthetic mixture
ARI vs demuxlet single best (excluding doublets called by each tool)	souporcell	1	1	1	n/a
	vireo	1	1	1	n/a
	scSplit	0.97	0.97	0.98	n/a
ARI vs truth (including doublets)	souporcell	n/a	n/a	n/a	0.99
	demuxlet	n/a	n/a	n/a	0.76
	vireo	n/a	n/a	n/a	0.98
	scSplit	n/a	n/a	n/a	0.94
ambient RNA	souporcell	2.70%	3%	2.70%	6.70%
	truth	n/a	n/a	n/a	5%
doublets	souporcell	338 (6.8%)	313 (6.4%)	323 (6.2%)	420 (6.0%)
	demuxlet	1537 (31)	1811 (37.5%)	1596 (31.2%)	1329 (18.7%)
	vireo	306 (6.2%)	287 (5.9%)	285 (5.5%)	311 (4.4%)
	scSplit	127 (2.6%)	107 (2.2)	102 (2%)	89 (1.2%)
	truth	n/a	n/a	n/a	451 (6.4%)
euts	souporcell	1033	1068	1177	829
	demuxlet	790	743	922	799
	vireo	1039	1074	1185	876
	scSplit	1076	1102	1211	856
	truth	n/a	n/a	n/a	817
nufh	souporcell	659	663	691	1192
	demuxlet	317	264	330	1037
	vireo	660	665	694	1211
	scSplit	707	721	766	1271
	truth	n/a	n/a	n/a	1183
babz	souporcell	739	727	731	2239
	demuxlet	522	459	504	1591
	vireo	742	734	740	2241

	scSplit	818	742	806	2263
	truth	n/a	n/a	n/a	2242
oaqd	souporcell	1680	1469	1592	1250
	demuxlet	1341	1172	1345	1227
	vireo	1590	1481	1609	1261
	scSplit	1587	1501	1600	1353
	truth	n/a	n/a	n/a	1247
ieki	souporcell	586	591	630	1138
	demuxlet	418	383	447	1090
	vireo	586	590	630	1160
	scSplit	610	659	659	1241
	truth	n/a	n/a	n/a	1133
unassigned	souporcell	2	1	2	5
	demuxlet	0	0	0	0
	vireo	2	1	1	13
	scSplit	0	0	0	0

Evaluation of clustering metrics for each tool on HipSci experimental mixtures as well as one representative synthetic mixture with 5% ambient RNA and 6% doublets.

Supp Table 3: Maternal/Fetal clustering.

	method	**placenta1**	**placenta2**	**decidua1**
ARI vs demuxlet single best (excluding doublets called by each tool)	souporcell	0.96	0.96	0.93
	vireo	0	0.18	0.3
	scSplit	0.03	0	0
ambient RNA	souporcell	4%	3.80%	6.80%
cells evaluated	souporcell	3835	3968	2119
	demuxlet	3805	3941	2115
	vireo	3835	3968	2119
	scSplit	3835	3968	2119
doublets	souporcell	49 (1.2%)	40 (1%)	3 (0.14%)
	demuxlet	189 (4.9%)	168 (4.2%)	112 (5.3%)
	vireo	660 (17.2%)	543 (13.7%)	229 (10.8%)
	scSplit	1705 (44%)	2297 (58%)	1145 (54%)
Maternal	souporcell	217	232	1944

	demuxlet	198	201	1863
	vireo	924	822	1240
	scSplit	1063	675	542
Fetal	souporcell	3316	3432	151
	demuxlet	3418	3572	140
	vireo	1427	1822	440
	scSplit	1067	996	432
unassigned	souporcell	253	274	21
	demuxlet	0	0	0
	vireo	824	781	210
	scSplit	0	0	0

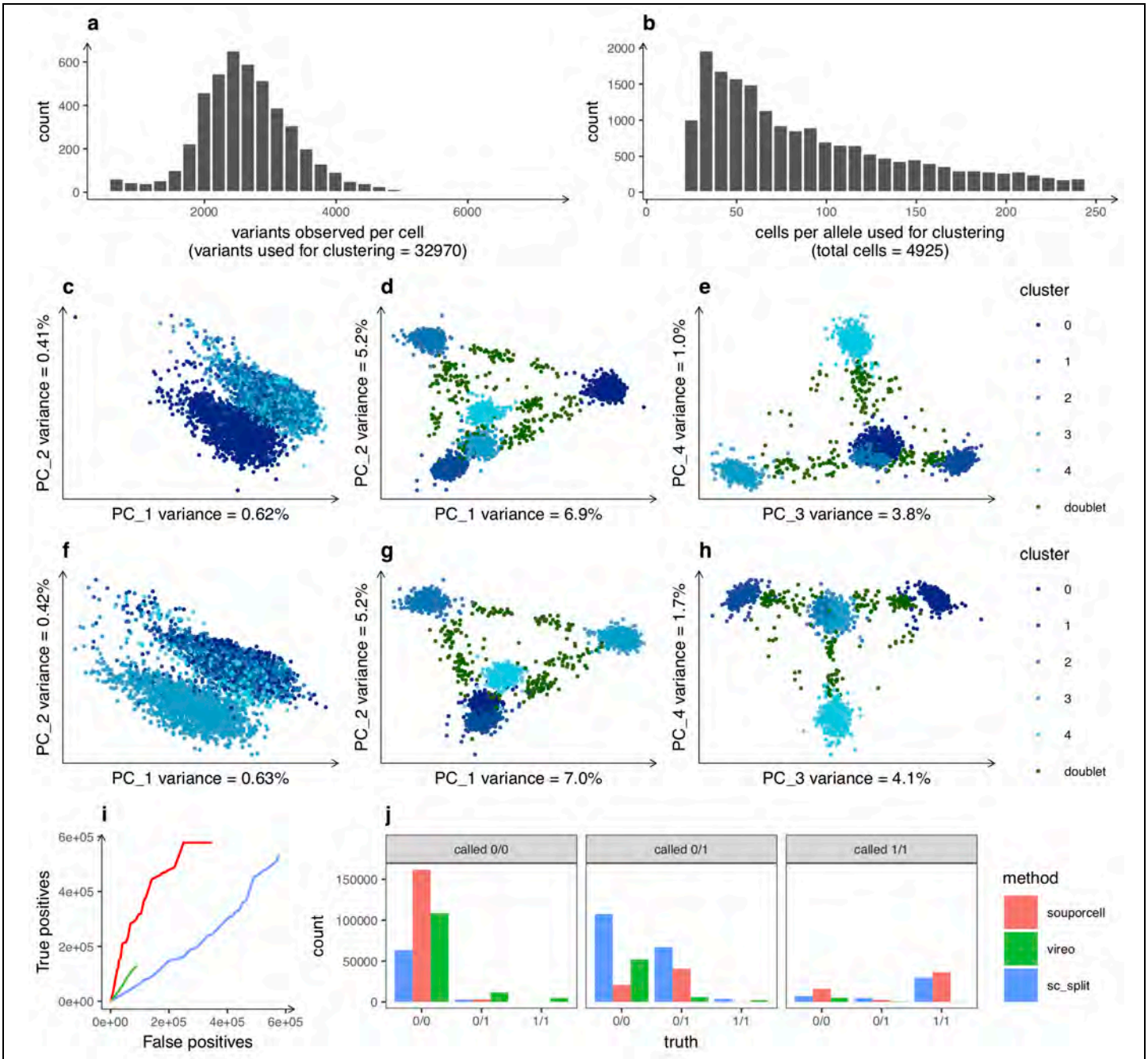
A comparison of the clustering performance of each tool on the maternal/fetal data.

Supp Table 4: Plasmodium clustering.

	method	**Plasmodium1**	**Plasmodium2**	**Plasmodium3***
ARI vs demuxlet single best (excluding doublets called by each tool)	souporcell	1	0.99	0.99
	vireo	0.95	0.51	0.24
	sc_split	0.6	0.46	0.45
ambient RNA	souporcell	2.4%	3.7%	6.8%%
doublets	sourporcell	92 (3.5%)	175 (9.2%)	41 (1.8%)
	demuxlet	621 (23.8%)	297 (15.7%)	283 (12.3%)
	vireo	241 (9.2%)	340 (18%)	423 (18.4%)
	sc_split	338 (13%)	272 (14.4%)	463 (20%)
3D7	sourporcell	1079	705	1805
	demuxlet	951	703	1649
	vireo	1040	476	883 (in 2 clusters)
	sc_split	1131 (in 2 clusters)	784 (in 2 clusters)	1390 (in 2 cluster)
GB4	sourporcell	392	268	166 (including the SenTh strains)
	demuxlet	331	242	73
	vireo	336 (in 2 clusters)	198	n/a
	sc_split	389	281 (cluster contains both	n/a

			GB4 and SenTh015)	
7G8	sourporcell	273	201	281
	demuxlet	223	183	229
	vireo	292	294 (cluster contains both 7G8 and SenTh028)	435 (including all non-3D7 strains)
	sc_split	237	311	440 (including all non-3D7 strains)
SenTh011	sourporcell	239	159	n/a
	demuxlet	128	139	11
	vireo	229	117	n/a
	sc_split	323	254 (cluster contains both Senth011 and a smattering of other cells)	n/a
SenTh015	sourporcell	215	149	n/a
	demuxlet	163	121	42
	vireo	no cluster represents	no cluster clearly represents this strain	n/a
	sc_split	190		n/a
SenTh028	sourporcell	323	232	n/a
	demuxlet	190	208	5
	vireo	320	no cluster clearly represents this strain	n/a
	sc_split	no cluster represents	split across many clusters	n/a
unassigned	sourporcell	6	23	0
	demuxlet	0	0	0
	vireo	150	335	551
	sc_split	0	0	0

A comparison of the clustering performance of each tool on the Plasmodium data.

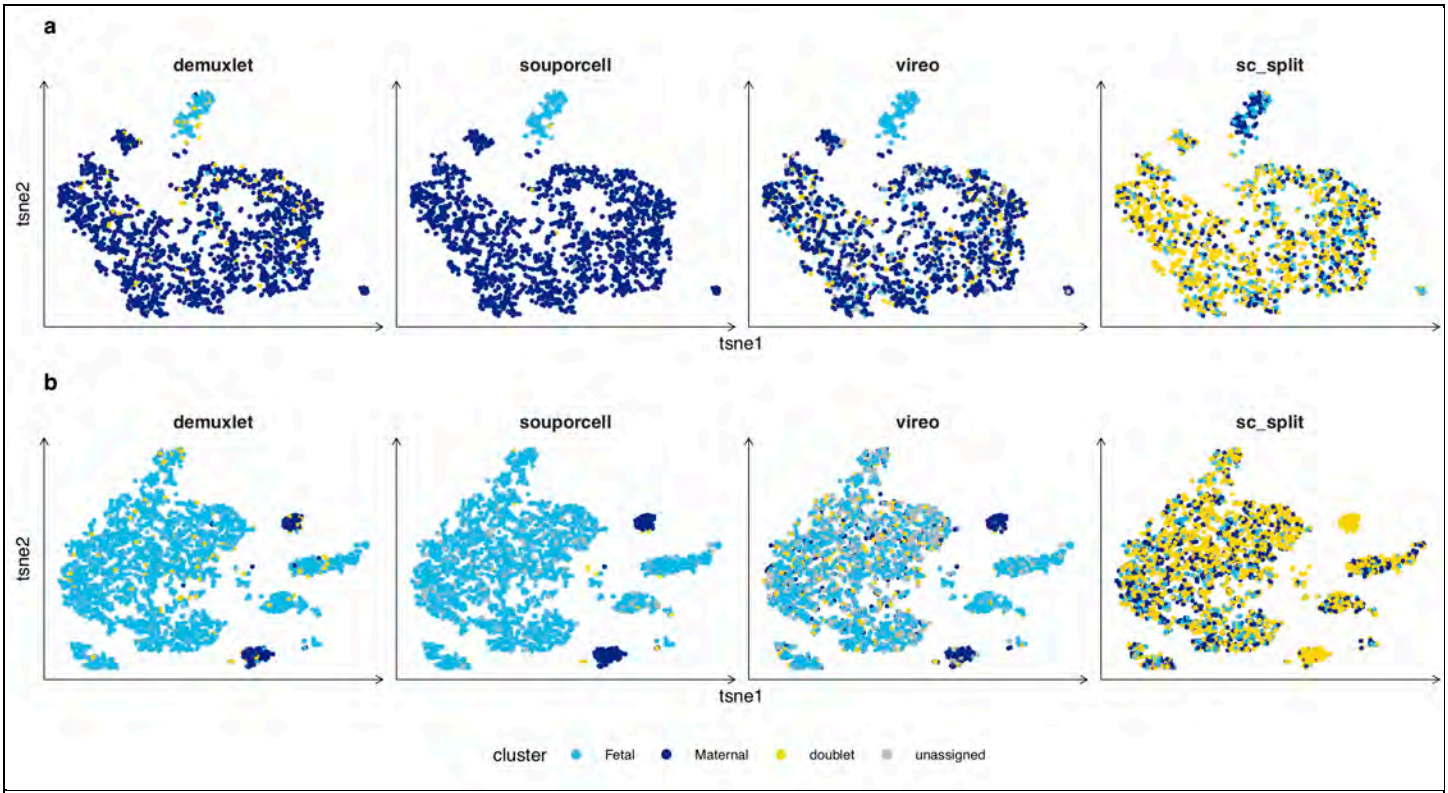


Supplementary Figure 1

HipSci data sparsity, replicates, and genotyping.

a, Distribution of the number of cells expressing a variant as well as **b,** the distribution of the number of alleles observed per cell that were used in souporcell clustering for HipSci mixture replicate 1 (replicates 2 and 3 are very similar, so not shown). **c,** Expression PCA of HipSci mixture replicate 2 (4832 cells) colored by genotype clusters from souporcell. **d,** and **e,** PCAs of the normalized cell-by-cluster loss matrix of HipSci mixture replicate 2 also colored by genotype cluster. **f,** Expression PCA of HipSci mixture replicate 3 (5144 cells) colored by genotype clusters. **g** and **h,** PCAs of normalized cell-by-cluster loss matrix of HipSci mixture replicate 3 colored by genotype cluster. **i,** Assessing genotype calling across souporcell, vireo, and scSplit. We plot true positive versus false positive genotype calls while sweeping the threshold on genotype likelihood. These are compared to a truth set obtained from variant calls on the WGS data **j,** Each method's genotype calls versus the true genotype of each tool for a synthetic mixture of five HipSci lines with 6% doublets and 10%

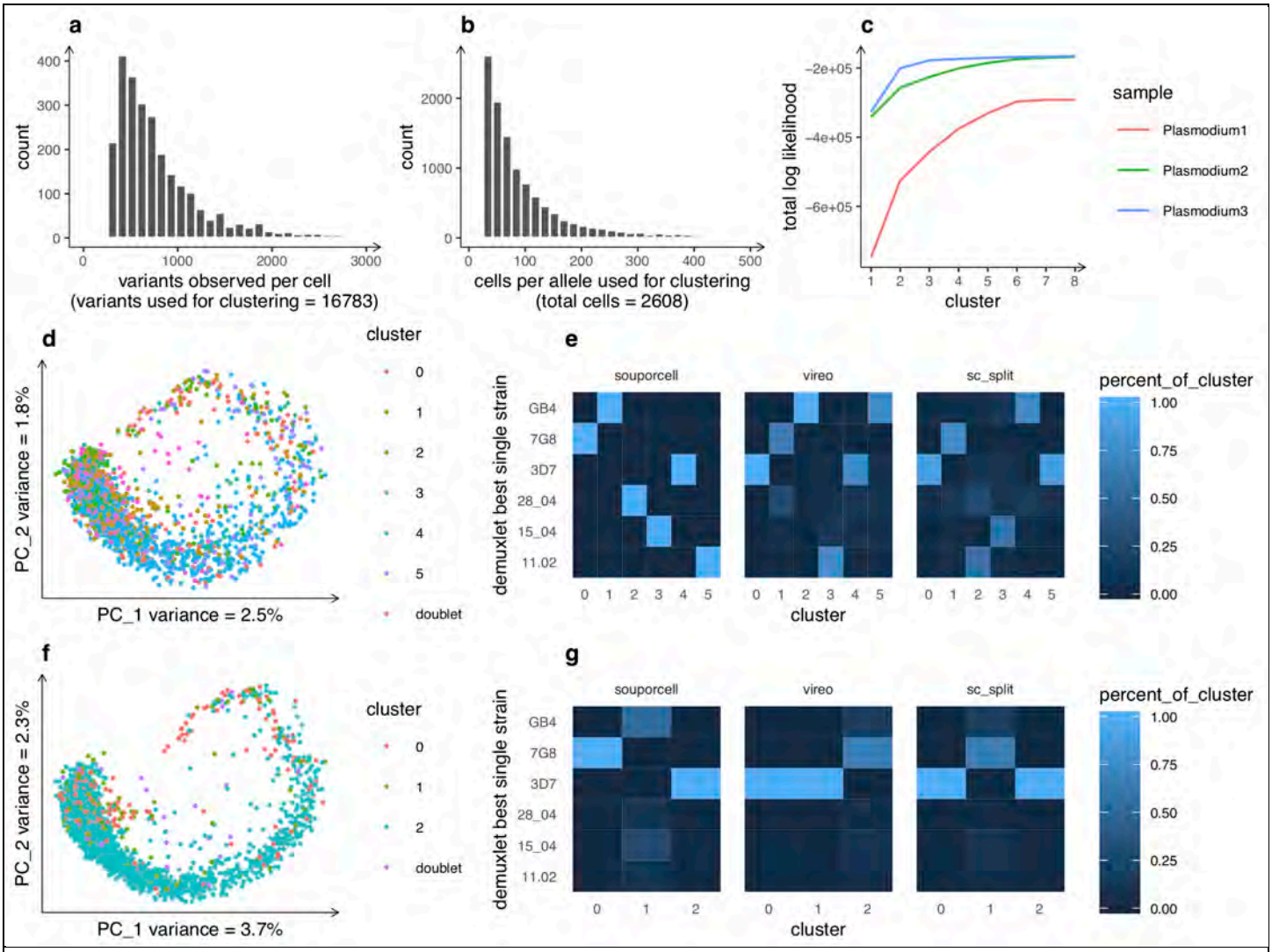
ambient RNA with a 0.95 probability threshold for each tool. The facets are the genotype calls made by each tool and the x-axis shows the correct assignments according to the WGS data. We observe that a major error mode for both vireo and scSplit compared to souporcell is that homozygous reference variants are mis-called as heterozygous because ambient RNA is not accounted for in these methods.



Supplementary Figure 2

Maternal/Fetal decidua1 and placenta2.

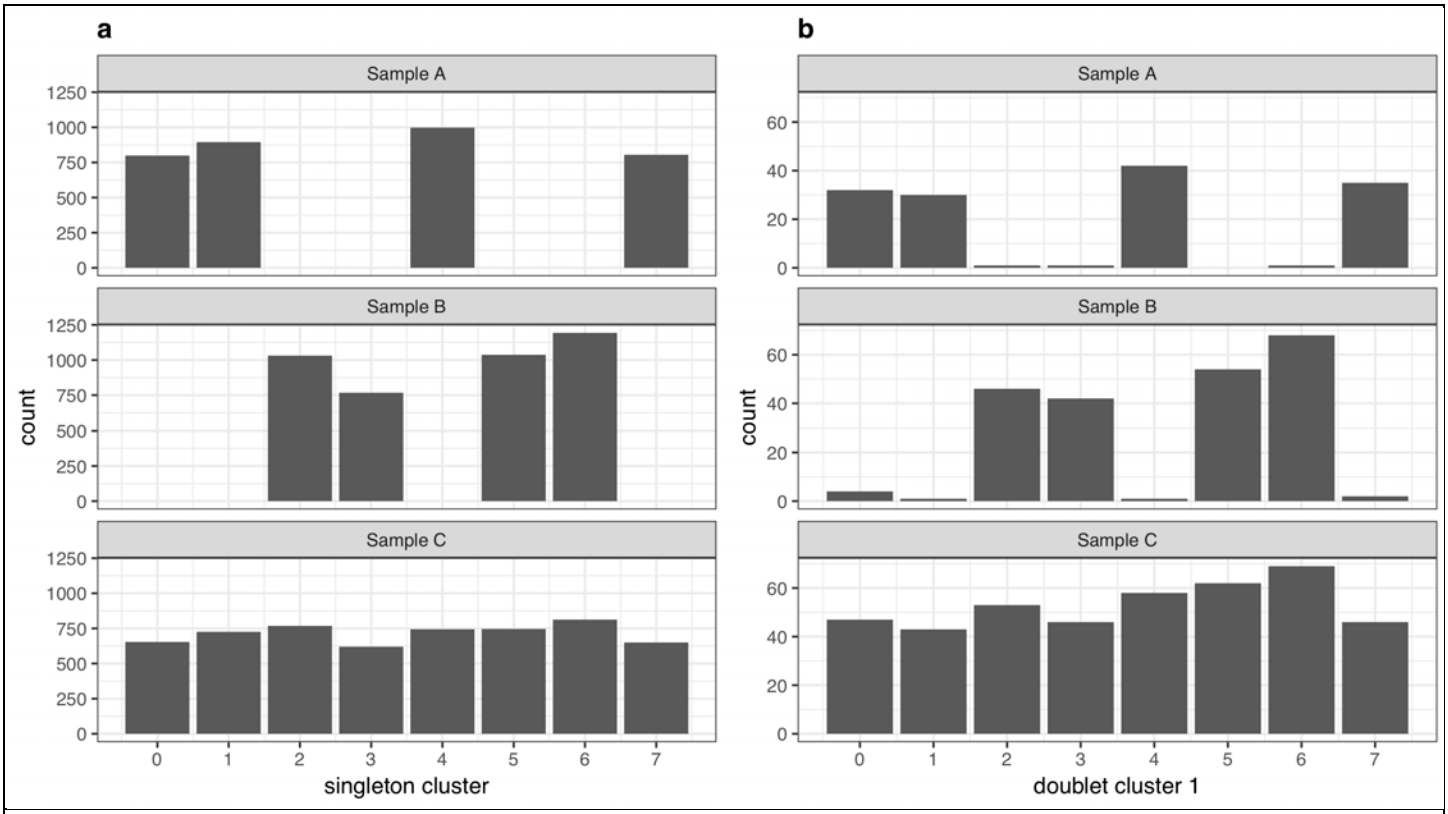
a, Expression t-SNE of a decidua1 sample (FCA747063, 2119 cells) colored by genotype clusters for each tool. Souporcell and demuxlet are highly concordant (ARI = 0.93). Vireo misidentifies a significant number of maternal cells as fetal cells. Excluding doublets and unassigned cells, vireo has an ARI of 0.3 versus demuxlet. scSplit has many errors resulting in an ARI versus demuxlet of 0. **b,** Expression t-SNE of placenta2 sample (3968 cells) colored by genotype clusters for each tool. Souporcell is again highly concordant with demuxlet (ARI = 0.96). Vireo has significant problems producing an ARI vs demuxlet of 0.18, even when excluding doublets and unassigned cells called by either tool. Like the other maternal/fetal samples, scSplit struggles and has and ARI versus demuxlet of 0.



Supplementary Figure 3

Plasmodium clustering.

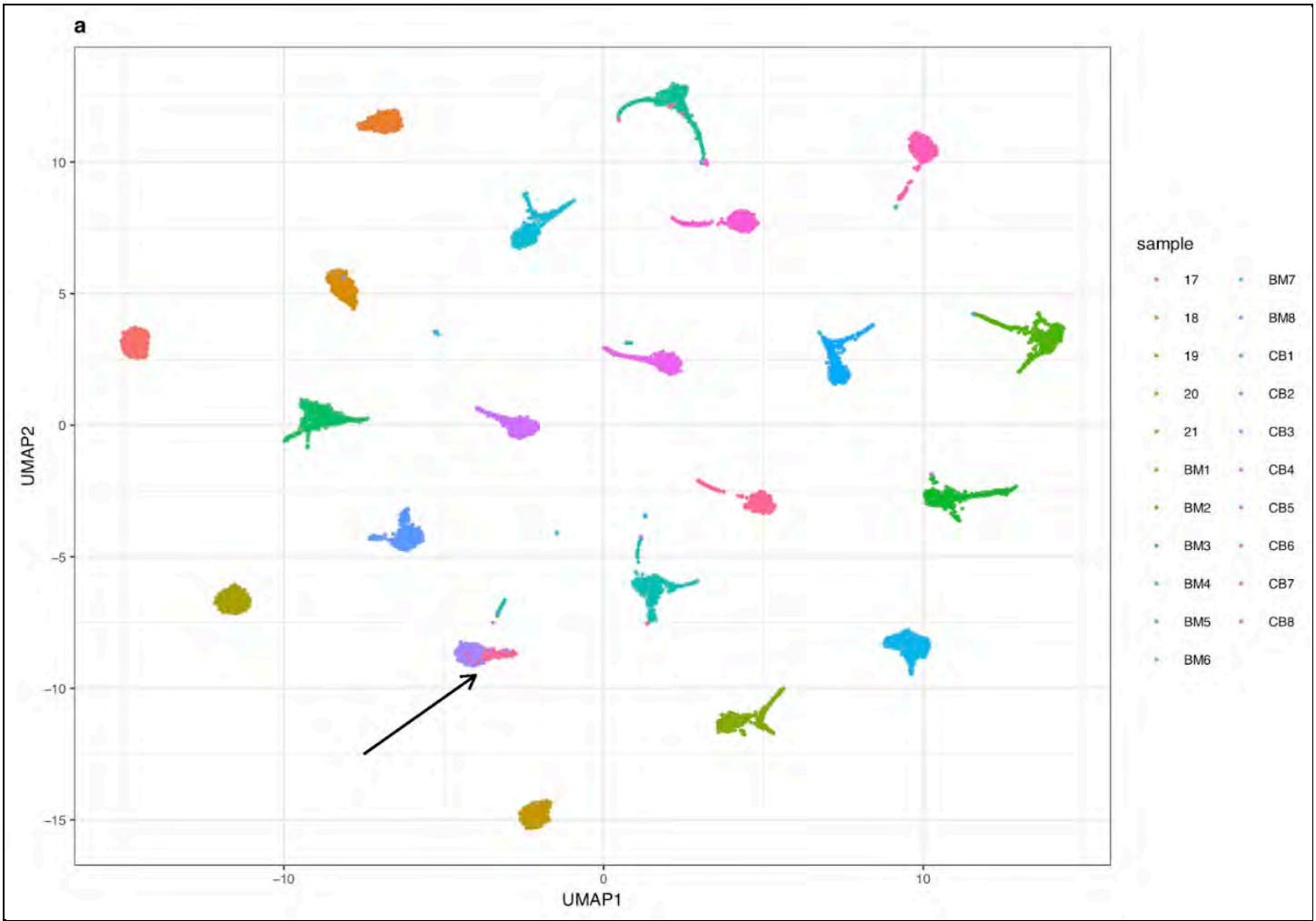
a, Distribution of number of variants observed per cell used for clustering (with at least 4 cells required to support each allele) and the total number of variants used for clustering on the Plasmodium1 sample. **b,** Distribution of counts of the number of cells expressing each allele used for clustering as well as the total number of cells in the Plasmodium1 sample. **c,** Elbow plots for each Plasmodium data set show relatively strong support for the correct number of clusters (6) for Plasmodium1, but less clear results for Plasmodium2, which suffered from higher amounts of ambient RNA, and for Plasmodium3, which due to more cell numbers biased towards three genotypes rather than a relatively even mixture. For this reason, we analyze Plasmodium3 with k=3. **d,** Expression PCA of the Plasmodium2 sample (1893 cells) colored by genotype clusters as called by souporcell. **e,** Confusion matrix heatmap of the demuxlet best single strain (Y axis) versus souporcell, vireo, and scSplit. For souporcell we see one cluster per strain as expected. Both vireo and scSplit have the majority strain, 3D7, split across two clusters and two other strains combined into a single cluster. **f,** Expression PCA of the Plasmodium3 sample (2293 cells) colored by genotype clusters as called by souporcell. **g,** Confusion matrix heatmap of the demuxlet best single strain (Y axis) versus souporcell, vireo, and scSplit genotype clusters with k=3. Souporcell clusters out the 3D7 and 7G8 strains correctly and puts all other cells into the final cluster while both vireo and scSplit put 3D7 into two clusters and all other cells into the remaining cluster.



Supplementary Figure 4

Demuxlet data

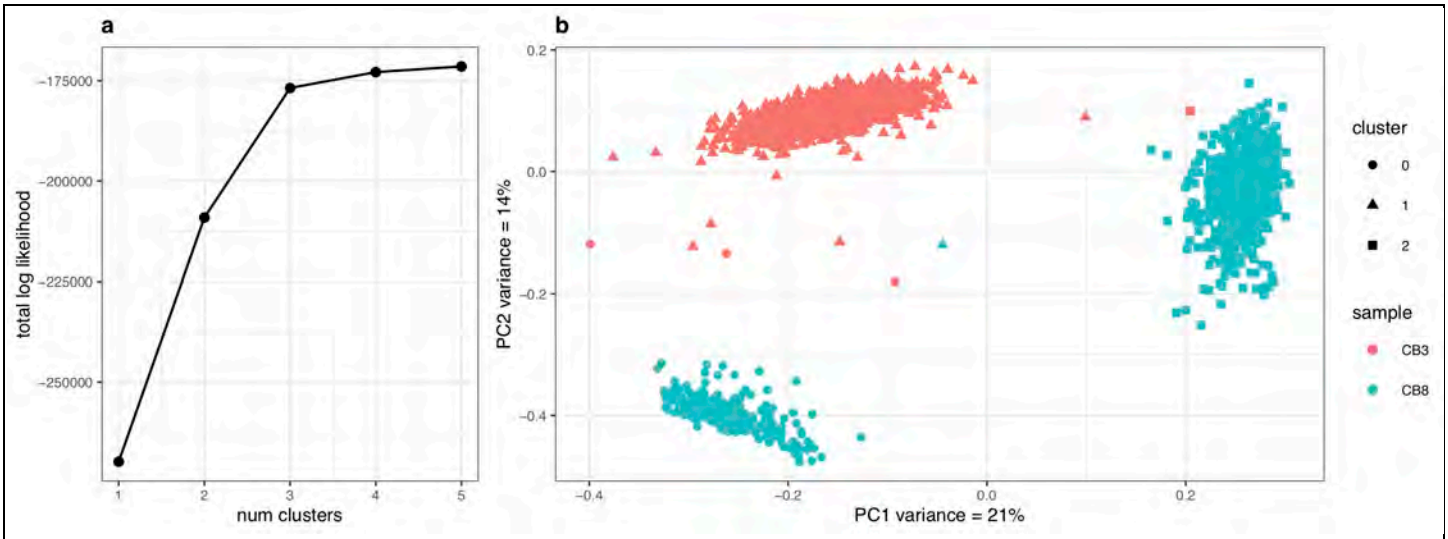
a, souporcell cluster assignments of singletons for combined dataset showing that Sample A and Sample B are non-overlapping and Sample C contains all 8 samples. **b,** shows the first cluster of the doublet assignment for doublets showing largely non-overlapping assignments between Samples A and B.



Supplementary Figure 5

21 donor synthetic mixture.

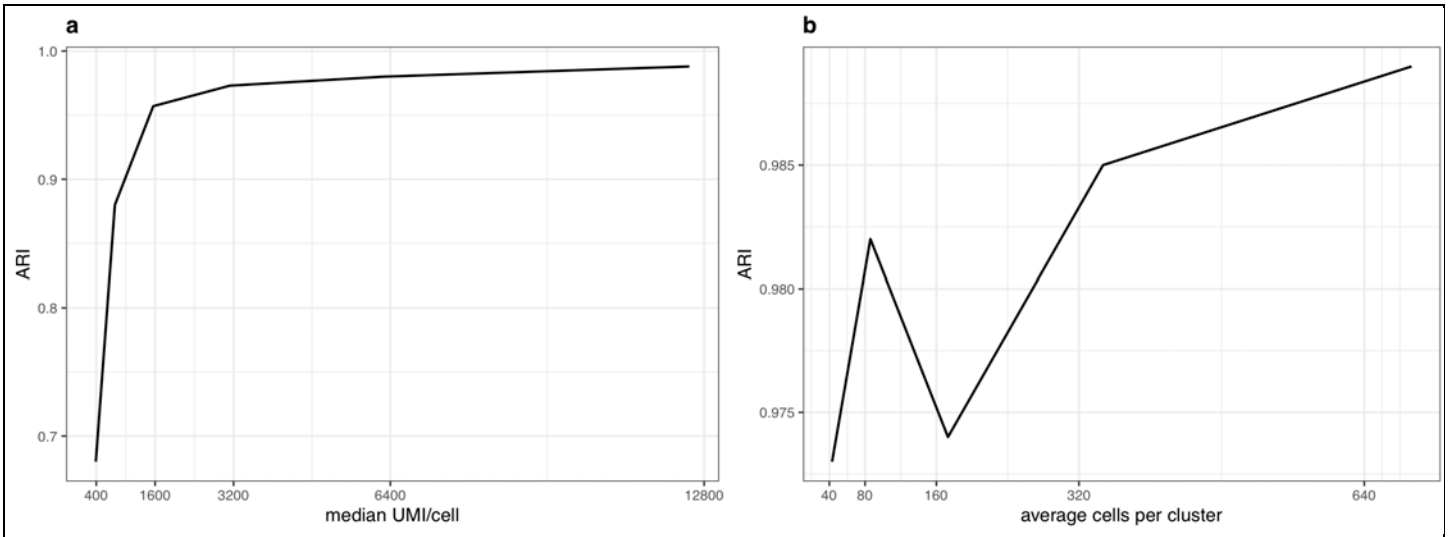
a, Umap of the normalized log likelihood cluster matrix for the singletons of a mixture of the 5 HipSci samples and the 16 PBMC samples from the Human Cell Atlas project. The main error is the assignment of 129 CB8 cells to the CB3 dominant cluster indicated by the arrow. We show later that this is likely due to contamination.



Supplementary Figure 6

Contamination of CB8 samples

a, Elbow plot of CB8+CB3 synthetic mixture with 3% doublets shows a clear preference for three clusters rather than the expected two.
b, Shows the PCA of the normalized cell by cluster log likelihood matrix (n=2716 cells) showing three distinct genotypes.



Supplementary Figure 7

UMI and Cell downsampling.

a, The synthetic mixture of 5 HipSci cell lines with 6% doublets and 5% ambient RNA with UMIs downsampled shows predominantly good clustering, but performance drops below 800 UMIs/cell. **b,** The clustering is consistently good with downsampled cells down to an average cell per cluster of 40. The cluster with the fewest cells in the 40 average cells per cluster had 20 cells.