SpotClean adjusts for spot swapping in spatial transcriptomics data

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Spatial transcriptomics is a powerful and widely used approach for profiling the gene expression landscape across a tissue with emerging applications in molecular medicine and tumor diagnostics. Recent spatial transcriptomics experiments utilize slides containing thousands of spots with spot-specific barcodes that bind RNA. Ideally, unique molecular identifiers (UMIs) at a spot measure spot-specific expression, but this is often not the case in practice due to bleed from nearby spots, an artifact we refer to as spot swapping. To improve the power and precision of downstream analyses in spatial transcriptomics experiments, we propose SpotClean, a probabilistic model that adjusts for spot swapping to provide more accurate estimates of gene-specific UMI counts. SpotClean provides substantial improvements in marker gene analyses and in clustering, especially when tissue regions are not easily separated. As demonstrated in multiple studies of cancer, SpotClean improves tumor versus normal tissue delineation and improves tumor burden estimation thus increasing the potential for clinical and diagnostic applications of spatial transcriptomics technologies.
Spatial transcriptomics (ST) is a powerful and widely used approach for profiling genome-wide gene expression across a tissue. In a typical ST experiment, fresh-frozen (or FFPE) tissue is sectioned and placed onto a slide containing spots, with each spot containing millions of capture oligonucleotides with spatial barcodes unique to that spot. The tissue is imaged, typically via Hematoxylin and Eosin (H&E) staining. Following imaging, the tissue is permeabilized to release RNA which then binds to the capture oligonucleotides, generating a cDNA library consisting of transcripts bound by barcodes that preserve spatial information. Data from an ST experiment consists of the tissue image coupled with RNA sequencing data collected from each spot. A first step in processing ST data is tissue detection, where spots on the slide containing tissue are distinguished from background spots without tissue. Unique molecular identifier (UMI) counts at each spot containing tissue are then used in downstream analyses (Supplementary Fig. 1).

Ideally, a gene-specific UMI at a given spot would represent expression of that gene at that spot. This is not the case in practice. As we demonstrate here, messenger RNAs bleed between and among nearby spots causing substantial contamination of UMI counts, an artifact we refer to as spot swapping. Spot swapping is related to, but distinct from, previously defined sources of contamination which have been widely recognized over the past decade in next-generation sequencing studies. A second type of contamination is contamination which have been widely recognized over the past decade in next-generation sequencing studies. Specifically, improvements in sequencing technologies have greatly increased the speed and scale at which data can be obtained, but the advantages rely on multiplexing where indexes (or barcodes) are attached to each RNA (or DNA) fragment in a sample prior to pooling so that sample-specific transcripts can be identified in the sequenced pool. In spite of the major advantages in reduced cost and increased efficiency, a disadvantage is that indexes from one sample may attach to transcripts from another, an error referred to as index hopping or barcode swapping. While present in most datasets, good statistical methods are in place to adjust for this type of contamination.

A second type of contamination is specific to single-cell RNA sequencing (scRNA-seq) experiments, where ambient RNA is sequenced along with RNAs from an individual cell. As with index hopping, robust statistical methods are in place to adjust for ambient RNA contamination in droplet-based scRNA-seq experiments.

While existing methods can be used to remove some sources of contamination present in an ST experiment, they are insufficient to adjust for effects due to spot swapping as they do not accommodate the spatial nature of the contamination (for further discussion, see Supplementary Section S1). In barcode swapping, the swapping takes place in the pooled cDNA library, and so a barcode from one sample has an equal chance of binding reads from any another sample. In spot swapping, barcodes from one spot are much more likely to bind reads from nearby spots. While barcode swapping might also affect an ST experiment during sequencing, spot swapping is a distinct type of contamination.

Below we demonstrate the effect of spot swapping in multiple ST experiments. While it is straightforward to quantify the extent of spot swapping from tissue spots to background spots, assessing the extent of spot swapping within tissue is challenging in most settings without prior information. Toward this end, we consider marker genes where expression is known to be high in particular tissue regions, and low in others. We also conduct a human–mouse chimeric experiment to evaluate the extent of human-specific transcripts in mouse regions, and vice versa.

To adjust for spot swapping in ST experiments, we propose a computational approach called SpotClean, implemented in the R package R/SportClean. Simulations and case study analyses show that SpotClean increases the specificity of marker gene expression, increases the power for identifying differentially expressed genes, improves the specificity of clusters, and increases the accuracy of spot annotations. The impact of these improvements in studies of breast, pancreatic, and colorectal cancer is also demonstrated.

Results

Spot swapping in public datasets. Figure 1 shows spot swapping from tissue to background in a study of human brain from Maynard et al. Specifically, Fig. 1b shows that UMI counts at background spots (which are zero in the absence of contamination) are far from zero, with the counts decreasing with increasing distance from the tissue. The distributions of total UMI counts in tissue and background spots show considerable overlap (Fig. 1c); and the expression patterns at tissue spots and nearby background spots are similar, but distinct from distant background spots, as shown for 50 genes in Fig. 1d. As a result of expression similarity between the tissue and nearby background, tissue and background spots often cluster together. This is emphasized in Fig. 1f, where spots on the slide are colored by membership in the graph-based clusters shown in Fig. 1e. As shown, many of the clusters contain spots from the tissue and nearby background. Supplementary Figs. 2–5 show similar results from 16 additional datasets; and Supplementary Table 1 shows that the proportion of UMI counts in background spots ranges from 5% to 20% in most datasets.

Figure 1, Supplementary Figs. 2–5, and Supplementary Table 1 demonstrate that spot swapping occurs from tissue to background. The effect is not explained by inefficient barcode binding, as might be observed in mitochondrial genes or long non-coding RNAs, for example (Supplementary Fig. 6), or by differences in permeabilization times (Supplementary Fig. 7). While spot swapping from tissue to background reduces expression levels at affected spots, a bigger concern is spot swapping from one tissue spot to another, as this confounds downstream analyses.

Evaluating the extent of spot swapping from tissue spot to tissue spot is challenging as it requires information about expected expression of specific genes at specific tissue locations. Toward this end, we first consider tissue-specific marker genes that identify distinct tissue layers in brain. In the absence of spot swapping, expression for a layer-specific marker should be high within that layer, and low (or off) in other layers. When spot swapping occurs, marker expression is relatively high in adjacent layers and decreases with increasing distance from the layer. This is evident with GFAP, for example, a marker known to be up-regulated in white matter (WM) and in the first annotated layer of the dorsolateral prefrontal cortex (Layer1). Supplementary Figure 8 shows high expression of GFAP in WM and Layer1 spots, as expected, but also relatively high expression in tissue spots adjacent to WM and Layer1, with GFAP expression decreasing as distance from WM (or Layer1) increases. While it is possible that some increase in marker expression in adjacent tissue spots may be due to the presence of WM (or Layer1) cells at those spots, we note that the rate of expression decay into the background spots (where no cells are present) is similar to the rate of decay into adjacent tissue regions. Consequently, the possible presence of WM (or Layer1) cells in adjacent tissue spots is not sufficient to fully explain the observed expression pattern. Similar results are shown for a WM marker, MOBP (Supplementary Fig. 8), as well as additional markers in multiple datasets (Supplementary Fig. 9).

A study of human breast cancer provides another example. Supplementary Fig. 10 shows expression for a highly specific breast cancer marker, ERBB2 (also called HER2). Because of its high specificity (it is typically expressed at a low level in normal breast tissue, but highly expressed in many breast tumors), ERBB2 is used in clinical practice as a target of a number of
therapies. Supplementary Fig. 10 shows high expression of ERBB2 in the tumor tissue, but also high expression in nearby normal tissue that decreases with increasing distance from the tumor. As mentioned above, the increased expression in adjacent normal tissue may be due to the presence of both tumor and normal cells in those spots. However, this is not sufficient to fully explain the effect as the rate of decay from tumor tissue to background, an effect that decreases as distance from the tissue increases. The positions of the three regions are shown in Supplementary Fig. 2.

Experimental validation of spot swapping using chimeric samples. To more directly quantify the extent of spot swapping, we conducted chimeric experiments where human and mouse tissues were placed contiguously during sample preparation. For each experiment, we annotated the H&E images to identify species-specific regions, and we calculated the proportion of mouse-specific reads in human spots and human-specific reads in mouse spots (Fig. 2, Supplementary Fig. 11). This is a lower bound on the proportion of spot-swapped reads (LPSS) as it does not account for spot swapping within species (e.g. reads from human spot bound by probes at human spot $t$), or for reads in the background. LPSS ranges between 10% and 15% in these experiments (Supplementary Table 1).

Taken together, results from a comparison of tissue and background expression (Fig. 1 and Supplementary Figs. 2–5), analysis of marker genes in brain and breast cancer tissue (Supplementary Figs. 8–10), and the chimeric experiment (Fig. 2, Supplementary Fig. 11, and Supplementary Table 1) demonstrate that spot swapping affects UMI counts in ST experiments. As we show below, this nuisance variability decreases the power and precision of downstream analyses.

SpotClean is a probabilistic model that adjusts for spot swapping. To adjust for the effects of spot swapping in ST experiments, we developed SpotClean. SpotClean is based on a probabilistic framework that accommodates spot-swapped reads to provide improved estimates of UMI counts for every gene at each spot. Specifically, SpotClean models gene-specific expression at a given spot as a function of reads present in tissue at that spot, reads that bleed out into other spots, and reads that bleed in from background. SpotClean improves estimates of UMI counts for every gene at each spot, thereby reducing the effects of spot swapping and improving the power and precision of downstream analyses.

Fig. 1 Spot swapping in human brain sample LIBD_151507. a H&E-stained image. Scale bar is 1 mm. b Unique molecular identifier (UMI) total counts in the background decrease with increasing distance from the tissue. Tissue and background spot annotations are taken from Maynard et al. The perimeter delineating tissue and background is shown in white; also shown in white are tissue spots originally called background in Maynard et al. that appear to contain tissue in the H&E image. The spots shown in white have been removed from the summaries shown in to ensure that the effects shown are not due to spots on the tissue-background boundary, or to tissue in the background. c UMI count densities for tissue and background spots show relatively high counts in the background. d Counts of the top 50 genes from a select tissue region (upper), from a nearby background region (middle), and from a distant background region (bottom) show the similarity between expression in tissue spots and nearby background spots due to spot swapping from tissue to background, an effect that decreases as distance from the tissue increases. The positions of the three regions are shown in Supplementary Fig. 2. e Graph-based clustering of all spots identifies 9 clusters, visualized in uniform manifold approximation and projection (UMAP) plot. f Spots on the slide are colored by their cluster membership shown in e. Black arrows highlight areas of spot swapping of signal from tissue to background.
In most cases, data processed via SpotClean genes known to be DE between these layers as discussed in Fig. 3b and Supplementary Fig. 12 show results for gold-standard WM and Layer6 using raw and SpotClean decontaminated data; to spots containing a mixture of the two species. Spots annotated as mixtures were removed prior to calculating the summaries in mouse spots; mouse-specific UMI counts in human spots). For the six boxplots from left to right, n = 2962, 751, 1014, 2962, 751, 1014 spots, respectively. The lower whisker, lower hinge, line inside box, upper hinge, and upper whisker represent the minimum, lower quartile, median, upper quartile, and maximum calculated without outlier values which are more than 1.5*inter-quartile range away from the hinges and are shown in separate dots. Note that there may be spot-swapped reads in these latter proportions (e.g. reads from human spot t bound by probes at human spot t'), but they cannot be identified in this experiment. The total UMI counts in human-specific genes and mouse-specific genes for HM-1. Similar plots for HM-2 and HM-3 are shown in Supplementary Fig. 11. Tissue spots on the perimeter as well as spots annotated as mixtures were removed prior to calculating the proportions in b in an effort to ensure that the effects shown are not due to spots on the tissue-background boundary.

Additional results are demonstrated in a study of breast cancer. Figure 4 shows expression for ERBB2 and MUC1, another breast cancer marker, before and after SpotClean. SpotClean increases specificity of these markers by maintaining expression in the tumor regions and reducing expression in the non-tumor regions. It also leads to improved separation of the tumor and non-tumor regions via clustering, as shown visually, and quantified by ARI scores. Similar results are shown in Fig. 5 in a study of pancreatic cancer.

SpotClean reduces the risk of overestimating malignancy in cancer studies. As the diagnosis and extent and invasiveness of a tumor is typically estimated through evaluation of an H&E image by a pathologist, there is now considerable interest in using ST experiments, which couple the H&E image with molecular profiling data, to improve diagnosis and precision therapy. ST can provide additional information by identifying subtle collections of malignant cells, but accurate spot annotation is required for this information to be useful in clinical practice, and especially so as not to overcall tumor burden. SpotClean demonstrates advantage toward this end. Figure 6a shows spots annotated using SpotClean data versus spots annotated using data that has not been decontaminated via SpotClean for the breast cancer sample discussed above. Compared with the H&E image annotations shown in Fig. 6a, which we consider to be a gold standard, the non-decontaminated data misidentifies many spots as malignant including those containing benign cells surrounding the tumor; the SpotClean decontaminated data more closely resembles identification of malignant cells on the H&E image. Specifically, over 13% of the spots are labeled malignant in the raw, but not other spots. Bleeding rates and the size of the neighborhood affected are estimated via gradient descent; latent expression levels are estimated using an EM algorithm.

SpotClean recovers true gene expression, provides more precise estimates of marker gene expression, and improves downstream analyses. Evaluations were conducted on simulated and case study data. In SimI, contaminated counts are generated assuming that local contamination follows a Gaussian kernel; SimII-IV relax the Gaussian assumption. Supplementary Tables 2–5 show the mean squared error (MSE) between true and decontaminated gene expression in simulated datasets; SpotClean provides better estimates of expression, reducing the MSE by over 20% in most simulations.

The benefits of SpotClean on marker gene estimation and on downstream analyses are also illustrated in case study data. Specifically, Fig. 3a shows that SpotClean improves the specificity of GFAP by maintaining expression levels in WM and Layer1 and reducing spurious expression in the other layers. Supplementary Fig. 9 shows similar results for four additional markers.

We also identified differentially expressed (DE) genes between WM and Layer6 using raw and SpotClean decontaminated data; Fig. 3b and Supplementary Fig. 12 show results for gold-standard genes known to be DE between these layers as discussed in Maynard et al. 9. In most cases, data processed via SpotClean results in increased fold-changes and smaller p-values, further suggesting that SpotClean results in more accurate expression estimates.
SpotClean decontaminated, data. Figures 6b and c show that expression in these questionably malignant spots is more similar to spots known to harbor non-malignant cells suggesting that these questionably malignant spots are false calls. Similar results are shown in Fig. 7 in a study of colorectal cancer where SpotClean decontaminated data leads to improved delineation of tumor and non-tumor regions as evidenced by enhanced tumor malignancy scores in tumor spots, and lower malignancy scores in non-tumor spots, compared with raw data (Fig. 7b). SpotClean also identifies a cluster not identified in previous work (SpotClean tumor cluster 1 shown in Fig. 7c); and multiple analyses suggest that this cluster is a distinct tumor sub-population containing both tumor and tumor-infiltrating immune cells. First, a careful look at the H&E stain shows that this group of spots is non-normal, but distinct from other tumor regions (red boxes, Fig. 7a). Second, 9 of the top 10 genes identified as DE between SpotClean tumor cluster 1 and other tumor clusters are immunoglobulin marker genes (from 74 total DE genes with adjusted p-value ≤ 0.01); and immunoglobulin expression for these 9 genes is largely specific to this cluster (Fig. 7d). Finally, the average malignancy score for this group is lower than other tumor clusters, but higher than normal spots, further suggesting that this group of spots contains both tumor cells and tumor-infiltrating immune cells (average malignancy scores at normal, tumor cluster 1, and other tumor spots are 0.384, 0.430, and 0.477, respectively). Taken together, this evidence suggests that cluster 1 identified by SpotClean maintains biologically relevant information and, in this case, provides for a more specific clustering that captures subtle structure present in the tissue.

**Discussion**

Common sources of contamination in next-generation sequencing experiments such as barcode swapping\(^4\)–\(^6\) and ambient RNA contamination\(^7,8\) have been widely recognized over the past decade (Supplementary Section S1). We here identify spot swapping, a related but distinct form of contamination present in the 10x Visium\(^1\), SpatialTranscriptomics\(^1\), and Slide-seqV2\(^2\) platforms. SpotClean adjusts for the effects of spot swapping using a probabilistic model that accommodates spot-swapped reads to provide improved estimates of gene-specific UMI counts at each spot. SpotClean may be used to obtain improved estimates of expression given data from the 10x Visium or SpatialTranscriptomics platforms; it is not applicable to platforms where background barcodes and/or accurate barcode positions are not provided (e.g. Slide-seqV2).

We have demonstrated the utility of SpotClean to adjust for spot swapping and, in doing so, to provide improved estimates of expression. Since the probability of a spot-specific barcode binding reads from another spot increases as the distance between spots decreases, most of the adjustments made by SpotClean are local (i.e. reads are reassigned from one spot to a nearby spot). Given this, SpotClean will have only a modest impact on some downstream analyses, but a more major impact on others. Specifically, since average expression within a region will remain largely unchanged post SpotClean, downstream analyses that rely on average expression (e.g. DE analyses) will show only slight improvements over the raw data, as shown here. Modest improvements can also be expected for data where clusters are easily separated. However, for more specific analyses and/or more subtle signals, the effects of SpotClean are greater. Specifically, SpotClean provides substantial improvements in marker gene analyses by decreasing expression in regions where markers are known to be lowly expressed, while maintaining expression levels in other regions. In addition, SpotClean substantially improves clustering results and spot annotations in situations where regions are not easily separated, which may have important implications for clinical applications of the ST technology (e.g. in cancer diagnosis and staging).
In summary, spatial transcriptomics provides unprecedented opportunity to address biological questions and enhance patient care, but artifacts induced by spot swapping must be adjusted for to ensure that maximal information is obtained from these powerful experiments. SpotClean provides for more accurate estimates of expression, thereby improving spot annotations and increasing the power and precision of downstream analyses.

**Methods**

**Versions.** The following software and packages were used in the analysis: R-4.0.2; R/SpotClean-0.99.0; R/SoupX-1.5.0; R/celda-1.5.11; R/Seurat-3.2.2; R/scran-1.17.20; R/SPOTlight-0.1.7; R/reticulate-1.16; Python-3.7.4; Python/spatialdie-1.1.3; bcfluastq v.2.20.0.422; FastQC-0.11.7; MultiQC-1.9. Space Ranger-1.2.2; Loupe Browser-4.2.0.

**SpotClean.** Let $K$ be the total number of spots, $G$ be the set of genes, $I_t$ be the set of tissue spots with cardinality $|I_t| = K_t$, and $I_b$ be the set of background spots with...
cardinality $|I_s| = K_s$ where $K_t + K_s = K$. $G$ defaults to genes that are highly expressed, highly variable, or both; this default can be relaxed by a user. The true (i.e., uncontaminated) UMI counts are given by $(Y_f^T)^{G \in I_s}$, and observed counts by $D = (X_f^T)^{G \in I_s \cup I_b}$. As our interest here is to characterize the extent of spot swapping, we introduce the missing variable $B_{g,t}$ to be the UMI count for gene $g$ leaving tissue spot $t$ and binding to tissue (or background) spot $j$. Likewise we define $S_j$ to be the UMI count arising from gene $g$ in tissue spot $t$ that remain at that spot and thus are not subject to bleeding. We decompose $Y_f^T$ into a sum: $Y_f^T = S_f + B_f$, where $B_f = \sum_{g \in I_s \cup I_b} B_{g,f}$ counts all bleed-outs from spot $t$ to other spots $k \neq t$. Extending notation, we set $Y_{f,b} = S_{f,b} = B_{f,b} = 0$ for background spots $b \in I_b$ since background spots do not express RNA. With these missing variables defined, we note that the measured count $X_{g,t} = S_{g,t} + B_{g,t}$, where $\sum_{g \in I_s \cup I_b} B_{g,t}$ represents UMI counts received at spot $j$ due to spot swapping. We leverage this missing-data formulation by flexibly modeling the component counts with independent Poisson distributions, which are known to be effective for UMI counts\cite{10.1038/s41467-022-30587-y}.

For a collection of spot and gene-specific parameters, as well as global parameters controlling the swapping rates, we parameterize the distributions as: $S_{g,t} \sim \text{Poisson}(\mu_{g,t} (1 - r_t))$ and $B_{g,t} \sim \text{Poisson}(\mu_{g,t} r_t ((1 - r_t)w_t + r_t \frac{1}{n}))$ where $r_t$ is the bleeding rate; $r_t$ is a distal and $1 - r_t$ is a proximal contamination rate. By taking the global bleeding rate $r_t \in [0, 1]$, it follows that the uncontaminated counts follow: $Y_f^T \sim \text{Poisson}(\mu_{g,f})$ for target parameters $\mu_{g,f}$, whose estimates constitute statistical estimates of the uncontaminated counts. Likewise for measured counts, $X_{g,t} \sim \text{Poisson}(\eta_{g,t})$, for induced gene and spot parameters. We define $w_t$ by a weighted Gaussian kernel: $w_t = K(d_{ij}, \sigma) / \sum K(d_{ij}, \sigma)$ where $d_{ij}$ is the physical Euclidean distance between spots $i$ and $j$ measured in pixels in the slide image, $\sigma$ is the kernel bandwidth, and $K(d, \sigma) = e^{-d^2/(2\sigma^2)}$ is a Gaussian kernel\cite{10.1038/s41467-022-30587-y}.

Parameter estimation. Plug-in estimates obtained by minimizing the residual sum of squares (RSS) between observed total counts and their expected values are used to estimate $f_{ij}$, $r_{ij}$, and $\alpha$. Specifically,

$$
\left(\hat{r}_j, \hat{r}, \hat{\alpha}, (\hat{\mu}_{i,t})_{t \in I_s} \right) = \arg\min_{r_j, r, \alpha, (\mu_{i,t})_{t \in I_s}} \sum \left(X_{g,t} - \eta_{g,t}\right)^2
$$

where $X_{g,t}, \eta_{g,t}, \mu_{g,t}$ are the summations of $X_{g,t}, \eta_{g,t}, \mu_{g,t}$ among all genes, respectively. To reduce computational complexity, $\tilde{\alpha}$ is taken as the minimum RSS calculated over a grid of candidate values. Explicit gradients are calculated for $r_j$ and $r_j$ and estimates are obtained by L-BFGS-B gradient descent\cite{10.1038/s41467-022-30587-y}. Details are provided in Supplementary Section S2. Since this optimization problem is not necessarily convex, it is important to choose appropriate initial values. For the initial values $(\mu^0_{i,t})_{t \in I_s}$ of $(\mu_{i,t})_{t \in I_s}$, we use the observed total UMI counts $(X_{g,t})_{t \in I_s}$ in tissue spots and scale them up so that they sum to the total UMIs in the data. The initial bleeding rate, $r^0_j$, is the average expression in background spots divided by the average expression in all spots; and the initial distal contamination rate, $d^0_j$, is
For each Slide-seqV2 dataset, we inspected total UMI counts of all spatial barcodes divided by average expression in all background spots.

Fig. 6 SpotClean improves identification of malignant spots in human breast cancer sample human_breast_2. a Malignant spot composition as estimated via SPOTlight is shown for the raw data (upper left) and SpotClean decontaminated data (upper middle). Scale bar is 1 mm. The raw data identifies many spots as malignant whereas the SpotClean decontaminated data more closely resembles the annotations derived from the H&E image (upper right). The insets highlighted in the upper panel are shown in the lower panel. b Spearman correlations between average expression in the malignant scRNA-seq cells and spot-specific expression were calculated. Boxplots of correlations are shown for \( n = 265 \) strongly non-malignant spots, 216 questionably malignant spots (spots labeled malignant in the raw data, but not the SpotClean decontaminated data), and 546 strongly malignant spots. The lower whisker, lower hinge, line inside box, upper hinge, and upper whisker represent the minimum, lower quartile, median, upper quartile, and maximum calculated without outlier values which are more than 1.5*IQR range away from the hinges and are shown in separate dots. Correlations with non-malignant scRNA-seq cells are also shown. The correlations show that expression in the questionably malignant spots more closely resembles that in non-malignant cells suggesting that the malignant classification in the raw data at these spots is likely false due to spot swapping. c The UMAP plot further demonstrates that the questionably malignant spots are also in the raw data are likely false positives as their expression more closely resembles that of non-malignant spots.

defined by average expression in the 25th–50th percentile of all background spots divided by average expression in all background spots.

With estimates \( \beta, \gamma, \sigma \) of the global parameters, true expression levels \( \mu_{fi} \) are readily estimated using an expectation-maximization (EM) algorithm. Details are provided in Supplementary Section S3. For the initial summations in all spots.

Estimation of spot-level contamination rate. For tissue spot \( t \), let \( c_t \) be the proportion of contaminated UMIs from total observed UMIs. We estimate \( c_t \) using the estimated contamination received in \( f_t \) over its estimated contaminated total counts from model fitting: \( c_t = 1 - \frac{\sum_{i} \frac{\mu_{fi}}{\gamma}}{\sum_{i} \frac{\mu_{fi}}{\gamma}} \).

Minimum number of background spots required for parameter estimation.

Given that the observed data is a single matrix with a fixed number of columns (spots), the number of unknown parameters is proportional to the number of tissue spots. In the extreme case where all spots are covered by tissue, we have more unknown parameters than observed data values. In this case the contaminated expressions are confounded with true expressions, and SpotClean estimation becomes unreliable. We recommend that the input data have at least 25% of spots not occupied by tissue, so that SpotClean has enough information from background spots to reliably estimate contamination.

Analysis of publicly available case study datasets. We downloaded UMI count matrices for 16 publicly available datasets, of which 13 came from 10x Visium, 1 came from SpatialTranscriptomics, and 2 came from Slide-seqV2. Links are provided in Supplementary Table 6. For each Visium and SpatialTranscriptomics dataset considered, the count matrix was normalized via scran, following the Seurat pipeline for dimension reduction, clustering, and visualization. Seurat functions FindVariableFeatures(\( \text{features} = 4000 \)), ScaleData(), RunPCA(), RunUMAP(), FindNeighbors(), and FindClusters() were applied under default settings. For each Slide-seqV2 dataset, we inspected total UMI counts of all spatial barcodes in the raw count matrix.

Evaluation of spot swapping in mitochondrial and long non-coding RNAs. To investigate potential differences in bleeding rates in mitochondrial genes and long non-coding RNAs (which may have less efficient barcode binding) vs. other genes, we calculated the proportion of UMI counts in background spots for each gene. Genes having UMI counts greater than 10 were divided into two groups: (1) mitochondrial genes and long non-coding RNAs, (2) the remaining genes. A two-sided Student’s t-test was conducted between the two groups for each dataset.

Identification of marker genes and DE genes in the DLPFC data. Maynard et al. consider spatial expression in the six-layered dorsolateral prefrontal cortex (DLPFC). The authors identified a number of marker genes for distinct layers of the DLPFC. In addition to these, we also considered marker genes from a single-cell RNA-seq study of Alzheimer’s disease where markers differentiating between known cell types were identified. The markers shown here were selected from these papers if they were highly expressed (in the upper 25th percentile) in the Maynard et al. datasets. We also evaluate the genes reported as DE between WM and Layer6 in Maynard et al. We filtered their list of DE genes and considered those genes having FDR \( \leq 10^{-4} \). From these, we chose the top 100 highest expression in the raw data, sorted by fold change, and selected the top 10 for each dataset. For the DE analysis, raw and decontaminated tissue matrices were normalized using scanr for each gene. \( p \)-values were obtained from a two-sample two-sided t-test between the 354 spots in WM and the 486 spots in Layer6. Summary statistics for the tests in Fig. 2b are reported in Supplementary Tables 7 and 8.

Human–mouse chimeric experiment. Fresh sections of normal human skin tissue were obtained from two participants (60-year-old and 39-year-old) after obtaining informed consent during routine Dermatologic surgery. Participants were not compensated. Human studies were conducted under a protocol (#2010-0367) approved by the School of Medicine and Public Health Institutional Review Board at the University of Wisconsin. The two participants in this study were randomly recruited in clinic on a single day during routine Dermatologic surgery. No other potential participants were approached because additional tissue was not needed. The participants consented to have their normal residual tissue from Dermatologic surgery used for this study. While this recruitment is biased toward patients who needed a Dermatologic procedure for non-melanoma skin cancer treatment, given that we intentionally sampled normal skin from these patients, we do not anticipate an impact on our results. The covariate-relevant population characteristics are as follows: 60-year-old and 39-year-old patients with current diagnoses of non-melanoma skin cancer requiring Dermatologic surgery for treatment.
Fresh mouse tissue was harvested on the same day. Animal studies were conducted under a protocol (#M5131) approved by the School of Medicine and Public Health Institutional Animal Care and Use Committee at the University of Wisconsin in compliance with policies established by the Office of Laboratory Animal Welfare at the National Institutes of Health. All animals were housed in a specific pathogen-free facility and fed a standard chow diet containing 4% fat. The set point for temperature was 72 °F and the set point for humidity was 30%. The dark/light cycle was 12:12.

Three chimeric tissue blocks were then prepared under cold conditions as follows and frozen over a bed of dry ice and stored at −80 °C in optimal tissue cutting (OCT) medium until they were ready to use:

- **HM-1**: Duodenum from a 10-week-old C57BL/6J male mouse as casing to a 4 mm punch section of human skin from a 60-year-old participant.
- **HM-2**: Colon from a 10-week-old C57BL/6J male mouse as casing to a 4 mm punch section of human skin from a 39-year-old participant.
- **HM-3**: Heart from a 10-week-old C57BL/6J male mouse encasing a 4 mm punch section of human skin from a 39-year-old participant.

The Visium Spatial Tissue Optimization Slide & Reagent kit (10X Genomics Cat no. 1000193) was used to optimize permeabilization conditions for the chimeric tissue according to manufacturer’s protocol and yielded an optimal tissue permeabilization time of 12 min. The Visium Spatial Gene Expression Slide & Reagent kit (10X Genomics Cat no. 1000184) was used to generate sequencing libraries. Sections were cut at 10 μm thickness and mounted onto Visium slide capture areas, stained with H&E, digitally imaged with an Aperio AT2 scanner, and then permeabilized for library preparation. Sequencing libraries were prepared following the manufacturer’s protocol. Initial quality control of the libraries was by analysis of 2 × 150 MiSeq data for each sample. The libraries were then sequenced on a NovaSeq 6000 (Illumina), with 29 bases from read 1 and 101 from read 2, at a depth of 500k–600k reads per spot. The actual depth was 455,652, 440,024, 538,709 reads per spot for sample HM-1, HM-2, HM-3, respectively.

### Alignment and pre-processing in the chimeric experiment

Raw FASTQ files were generated using bcl2fastq (Illumina, Inc.). The sequencing quality of each sample was evaluated using FastQC [26] and MultiQC [27]. All FASTQ files passed quality control. Tissue images were manually aligned using the Loupe Browser. Reads were aligned to the GRCh38-p10 reference genome (refdata-gex-GRCh38-p10-2020-A from https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest) and gene expression was quantified.
using Space Ranger under default parameters. Following alignment, we considered only those reads labeled confidently mapped by SpaceRanger; confidently mapped reads were those that mapped uniquely to a gene. We refer to genes in a human if it has prefix GRCh38; a mouse gene has prefix mm10. UMI counts were normalized for differences in total counts across species by scaling total UMI counts in mouse to match total UMI counts in human. Genes having average expression <0.01 were removed.

**Human and mouse tissue spot annotation in the chimeric experiment.** Tissue spots were labeled as human, mouse, or histopathological mixture based on visual inspection of the H&E images. A histopathological mixture spot is one with tissue contributions from both species that can be visually verified in the H&E-stained image. A pure human or pure mouse spot was relabeled as a computational mixture if the total UMI counts from human (mouse) exceeded the median of total UMI counts across all mouse spots (human spots). Background spots are defined as those spots on the slide outside the tissue region (not annotated as human, mouse, or mixture). Both histopathological or computational mixture spots were removed prior to analyses in an effort to ensure that the effects shown are not due to spots containing a mixture of the two species.

**Lower bound on the proportion of spot-swapped reads (LPSS).** Spot-swapped reads include reads from one tissue spot binding background probes (tissue-to-background) as well as reads at one tissue spot binding probes at another tissue spot (tissue-to-tissue). It is not possible to directly measure tissue-to-tissue swapping in this case; however, the chimeric experiment provides some insight into the extent of spot swapping tissue-to-tissue. We define LPSS in the chimeric experiment as the proportion of misclassified reads (mouse reads in human spots and human reads in mouse spots). This is a lower bound as it does not account for spot swapping within species (e.g. reads from human spot bound by probes at human spot) or for reads in the background.

**Cell type decomposition using SPOTlight.** For cell type decomposition, we applied SPOTlight to the Visium human breast cancer data and the Visium colorectal cancer single-cell RNA-seq data from Li et al.24. SPOTlight22 was applied under default settings. Parameters for SPOTlight are found at https://github.com/zijianni/codes_for_SpotClean_paper and will be submitted to Bioconductor. Codes for simulation and case study data analyses can be found at https://github.com/zijianni/codes_for_SpotClean_paper.

**Code availability**

The R package SpotClean is available at https://github.com/zijianni/SpotClean and will be submitted to Bioconductor. Codes for simulation and case study data analyses can be found at https://github.com/zijianni/codes_for_SpotClean_paper.

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**References**

1. Ståhl, P. L. et al. Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. Science 353, 78–82 (2016).
2. Stickels, R. R. et al. Highly sensitive spatial transcriptomics at near-cellular resolution with Slide-seq 3V2. Nat. Biotechnol. 39, 313–319 (2021).
3. Kircher, M., Sawyer, S. & Meyer, M. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. Nucleic Acids Res. 40, e3 (2012).
4. Griffiths, J. A., Richard, A. C., Bach, K., Lun, A. T. L. & Marioni, J. C. Detection and removal of barcode swapping in single-cell RNA-seq data. Nat. Commun. 9, 1–6 (2018).
5. Larsson, A. J. M., Stanley, G., Sinha, R., Weissman, I. L. & Sandberg, R. Computational correction of index switching in multiplexed sequencing libraries. Nat. Methods 15, 305–307 (2018).
6. Costello, M. et al. Characterization and remediation of sample index swaps by non-redundant dual indexing on massively parallel sequencing platforms. BMC Genomics 19, 1–10 (2018).
7. Young, M. D. & Bejbbati, S. SoupX removes ambient RNA contamination from droplet-based single-cell RNA sequencing data. Gigascience 9, 1–10 (2020).
8. Yang, S. et al. Decontamination of ambient RNA in single-cell RNA-seq with DeconX. Genome Biol. 21, 57 (2020).
9. Maynard, K. R. et al. Transcriptome-scale spatial gene expression in the human dorsolateral prefrontal cortex. Nat. Neurosci. 24, 425–436 (2021).
10. Browne, B., O’Brien, N., Murphy, C., J. & O’Donovan, N. HER-2 signaling and inhibition in breast cancer. Curr. Cancer Drug Targets 9, 419–457 (2009).
11. Oh, D.-Y. & Bang, Y.-J. HER2-targeted therapies for breast cancer. Nat. Rev. Clin. Oncol. 16, 1190–1208 (2019).
12. Moncada, R. et al. Integrating microarray-based spatial transcriptomics and single-cell RNA-seq reveals tissue architecture in pancreatic ductal adenocarcinomas. Nat. Biotechnol. 38, 333–342 (2020).
13. Kim, T. H., Zhou, X. & Chen, M. Demystifying ‘drop-outs’ in single-cell UMI data. Genome Biol. 21, 196 (2020).
14. Chung, M. K. Gaussian kernel smoothing. arXiv preprint arXiv:2007.09539 (2021).
15. Byrd, R. H., Lu, P., Nocedal, J. & Zhu, C. A limited memory algorithm for bound constrained optimization. SIAM J. Sci. Comput. 16, 1190–1208 (1995).
16. Dempster, A. P., Laird, N. M. & Rubin, D. B. Maximum likelihood from incomplete data via the EM algorithm. J. R. Stat. Soc. Ser. B (Methodol.) 39, 1–37 (1977).
17. Lun, A. T., Bach, K. & Marioni, J. C. Pooling across cells to normalize single-cell RNA sequencing data with many zero counts. Genome Biol. 17, 75 (2016).
18. Sturt, T. et al. Comprehensive Integration of Single-cell data. Cell 177, 1888–1902.e21 (2019).
19. Mathys, H. et al. Single-cell transcriptomic analysis of Alzheimer’s disease. Nature 570, 332–337 (2019).
20. Andrews, S., Krueger, F., Seidemann, A., Cutrone, F. & Kelly, S. FastQc: a Quality Control Tool for High Throughput Sequence Data, Vol. 11 (Babraham Bioinformatics, Babraham Institute, 2015).
21. Ewels, P., Magnusson, M., Lundin, S. & Keller, M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32, 3047–3048 (2016).
22. Elousa-Bayes, M., Nieto, P., Mereu, E., Gut, I. & Heyn, H. SPOTlight: seeded drop-outs in single-cell UMI data. Bioinformatics 32, 3047–3048 (2016).
23. Chung, M. K., et al. Spatial transcriptomics data. Nucleic Acids Res. 49, e50–e50 (2021).
24. Li, H. et al. Reference component analysis of single-cell transcriptomes elucidates cellular heterogeneity in human colorectal tumors. Nat. Genet. 49, 708–718 (2017).
25. Zhao, E. et al. Spatial transcriptomics at subspot resolution with BayesSpace. Nat. Biotechnol. 39, 1375–1384 (2021).
26. Svensson, V., Teichmann, S. A. & Stegle, O. SpatialDAE: Identification of spatially variable genes. Nat. Methods 15, 343–346 (2018).
27. Ni, Z. Processed data for “SpotClean adjusts for spot swapping in spatial transcriptomics data.” https://doi.org/10.5281/ZENODO.6478458 (2022).
28. Ni, Z. zijianni/SpotClean: assign DOI to this repo for publication. https://doi.org/10.5281/ZENODO.6478607 (2022).
29. Ni, Z. zijianni/codes_for_SpotClean_paper: assign DOI to this repo for publication. https://doi.org/10.5281/ZENODO.6478604 (2022).

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Author contributions

Z.N. discovered the spot swapping artifact. Z.N. and C.K. designed the research and wrote the first version of the manuscript. Z.N., C.K., and M.A.N. developed the SpotClean method. A.P. and R.B.H. designed the chimeric samples and conducted the chimeric experiments. Z.N. and S.C. conducted simulations and quality control evaluations. Z.N., S.C. and C.K. built and tested the R package. All authors contributed to writing the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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