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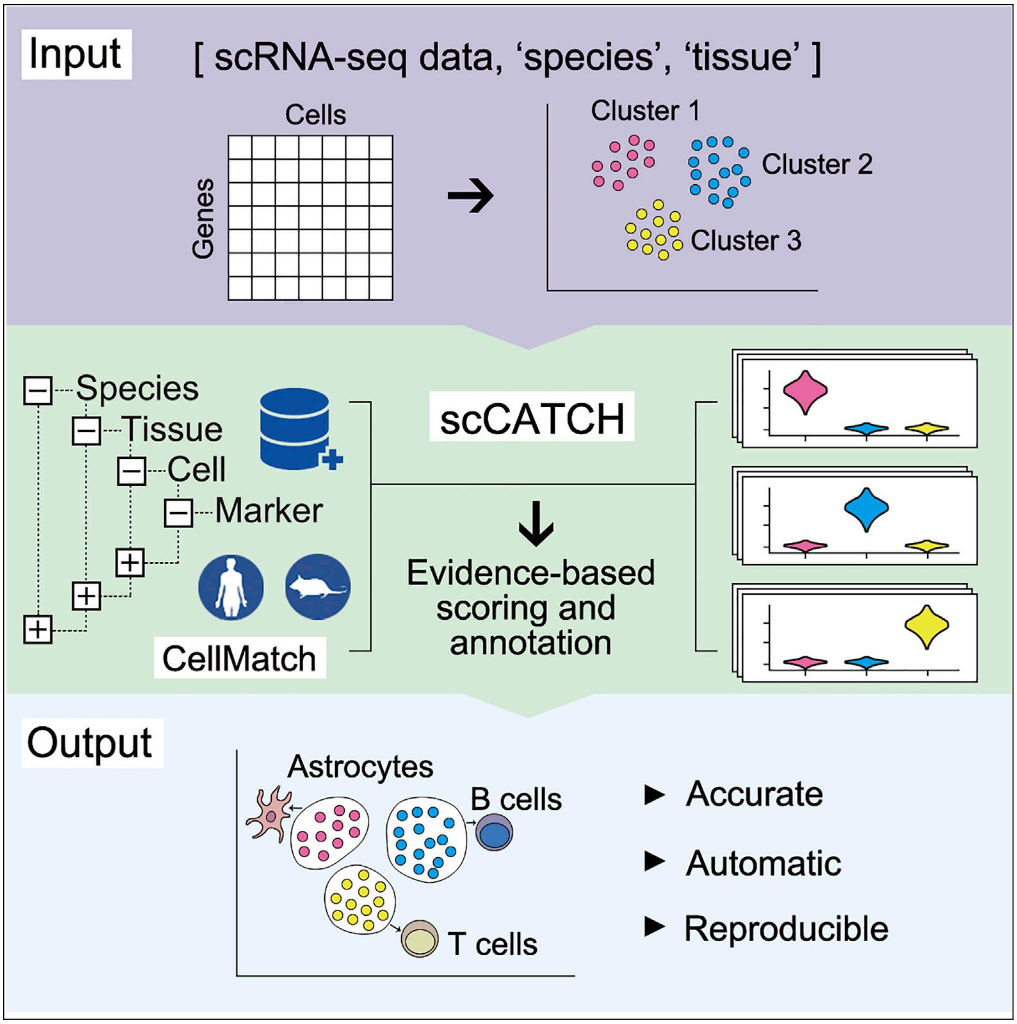

Xin Shao, Jie Liao, Xiaoyan Lu, Rui Xue, Ni Ai, Xiaohui Fan

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HIGHLIGHTS

Construction of a comprehensive tissue-specific reference database of cell markers

Paired comparisons to identify potential marker genes for clusters to ensure accuracy

Evidence-based scoring and annotation for clustered cells from scRNA-seq data

Accurate and replicable annotation on cell types of clusters without prior knowledge

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scCATCH: Automatic Annotation on Cell Types of Clusters from Single-Cell RNA Sequencing Data

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SUMMARY

Recent advancements in single-cell RNA sequencing (scRNA-seq) have facilitated the classification of thousands of cells through transcriptome profiling, wherein accurate cell type identification is critical for mechanistic studies. In most current analysis protocols, cell type-based cluster annotation is manually performed and heavily relies on prior knowledge, resulting in poor replicability of cell type annotation. This study aimed to introduce a single-cell Cluster-based Automatic Annotation Toolkit for Cellular Heterogeneity (scCATCH, https://github.com/ZJUFanLab/scCATCH). Using three benchmark datasets, the feasibility of evidence-based scoring and tissue-specific cellular annotation strategies were demonstrated by high concordance among cell types, and scCATCH outperformed Seurat, a popular method for marker genes identification, and cell-based annotation methods. Furthermore, scCATCH accurately annotated 67%–100% (average, 83%) clusters in six published scRNA-seq datasets originating from various tissues. The present results show that scCATCH accurately revealed cell identities with high reproducibility, thus potentially providing insights into mechanisms underlying disease pathogenesis and progression.

INTRODUCTION

Recent advancements in single-cell RNA sequencing (scRNA-seq) have furthered the understanding of heterogeneous cell compositions in complex tissues through the characterization of different cell types based on gene expression levels, thus facilitating our understanding on spatiotemporal biological phenomena or disease pathogeneses, cellular lineages or differentiation trajectories, or cell-cell communication (Haque et al., 2017; Macosko et al., 2015; Potter, 2018; Regev et al., 2017). In the data processing protocols of scRNA-seq experiments, cell type identification is a vital step for subsequent analysis, and two types of strategies have been reported, e.g., cell-based and cluster-based annotation (Abdelaal et al., 2019). For cell-based strategy, the similarities between cell-based data and reference cell databases are taken to determine potential cellular identities. Several methods including SingleR (Aran et al., 2019), CellAssign (Zhang et al., 2019a), Garnett (Pliner et al., 2019), scMap (Kiselev et al., 2018), and CHETAH (de Kanter et al., 2019) belong to this category. Cluster-based strategies perform cell type identification using differentially expressed marker genes at the level of pre-computed clusters. Experimentally validated cell markers through fluorescence-activated cell sorting (FACS), *in situ* hybridization, and immunohistochemistry (IHC) are often used as reference.

The major challenge of cell-based strategy lies in the determination of cell types on each cluster as multiple cells with different types are present in one cluster. As shown in Figure S1, cellular composition in each cluster could vary a lot. According to cell type annotation by SingleR, cluster 3 of Chen dataset was composed of 31.6% proximal tubule cells, 36.8% intercalated cells, and 31.6% principle cells. In this case, it is rather difficult to assign an accurate cell label to this cluster. For cluster-based analysis, the selection of cluster marker genes is critical for the sensitivity and selectivity of cell type determination. In Seurat (Butler et al., 2018), a widely used data processing pipeline of scRNA-seq studies, one-against-all methods are used to derive cluster marker genes. Inevitably, in this list, a bunch of pseudo marker genes (significantly upregulated in at least two clusters rather than in one cluster) may occur, which would lead to incorrect cell type annotation. Furthermore, prior knowledge on known cell markers is needed during manual match with cluster marker genes derived in previous step. Another level of uncertainty is introduced by the fact that one cell type is commonly associated with multiple cell markers and one cell marker can be linked with multiple cell types (Zhang et al., 2019b). Replicability of this cell annotation protocol could be further reduced with increased number of clusters and multiple selections of cluster marker genes.

To address these issues, a single-cell Cluster-based automatic Annotation Toolkit for Cellular Heterogeneity (scCATCH) is introduced here, in which cell types are annotated through the tissue-specific cellular taxonomy reference database (CellMatch) and the evidence-based scoring (*ES*) protocol (workflow presented

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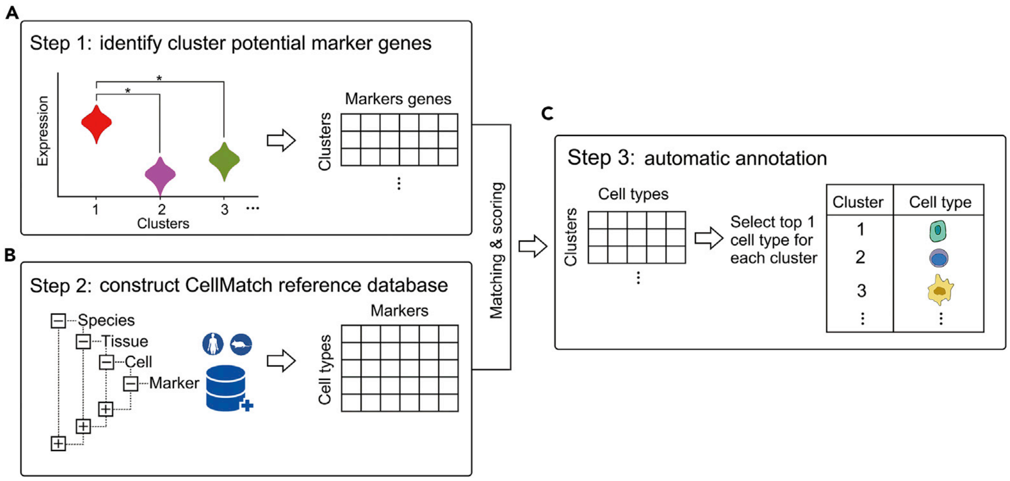
A
Step 1: identify cluster potential marker genes
Expression
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Step 2: construct CellMatch reference database
Species
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Matching & scoring
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Step 3: automatic annotation
Cell types
Clusters
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Select top 1 cell type for each cluster
Cluster
Cell type
1
2
3
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Figure 1. Automatic Annotation on Cell Types of Clusters from scRNA-Seq Data Using scCATCH

(A) Paired comparison of clusters to identify the potential marker genes for each cluster. Compared with every other cluster, genes significantly upregulated in only one cluster (log10 fold change $\geq$0.25, $p < 0.05$) and expressed in more than a quarter of cells ($\geq$25%) would be considered marker genes. p values were obtained through the Wilcoxon test. * indicates $p < 0.05$.

(B) Construction of tissue-specific cell taxonomy reference databases (CellMatch) with tissue-specific cell markers reported in the literature from humans or mice.

(C) Evidence-based score and annotation. For each cluster, cell types were scored on the basis of validated marker genes and their supporting literature, and the cell type with the highest score (top 1) was determined for the cluster.

in Figure 1). The performance of scCATCH was evaluated by cell identity benchmark datasets originating from three different tissues. We further validated the accuracy of scCATCH with six independent scRNA-seq datasets. Results indicated that scCATCH facilitates analysis on scRNA-seq data and provides novel insights into the mechanisms underlying disease pathogenesis and progression.

RESULTS

Validation of scCATCH Using the Benchmark scRNA-Seq Datasets

Knowledge in CellMatch reference database was derived from various resources, such as CellMarker (Zhang et al., 2019b), MCA (Han et al., 2018), CancerSEA (Yuan et al., 2019), and the CD Marker Handbook. In this reference database, cells were classified into three levels of subtypes in accordance with histological origin, expression of specific markers, or degrees of differentiation. Accordingly, a panel of 353 cell types and related 686 subtypes associated with 184 tissue types, 20,792 cell-specific marker genes, and 2,097 references of humans and mice were introduced into scCATCH as the reference database.

To validate the results of scCATCH, three independent scRNA-seq datasets, which were not recorded in the CellMatch database, were used, and cell types in these three datasets were identified or validated via FACS, *in situ* hybridization, or IHC. In particular, the Chen dataset (Chen et al., 2017) includes 203 mouse kidney cells and 3 cell types, namely intercalated cells, principal cells, and proximal tubule cells. The Xin dataset (Xin et al., 2016) includes 1,600 human pancreatic islet cells and 4 cell types, namely beta cells, alpha cells, delta cells, and pancreatic polypeptide (PP)-secreting cells. The Gierahn dataset (Gierahn et al., 2017) includes 3,694 human peripheral blood cells, namely B cells, T cells, dendritic cells (DCs), natural killer (NK) cells, and monocytes.

The cell types annotated by scCATCH were highly concordant with those verified from the literature for kidney cells, pancreatic islet cells, and peripheral blood cells (Figure 2). For the Chen dataset, scCATCH analysis identified intercalated cells and principal cells as collecting duct intercalated cells and collecting duct principal cells (Figure 2A), respectively, which is consistent with the organ origin of Chen dataset as renal collecting duct. For pancreatic islet cells in the Xin dataset, scCATCH accurately assigned cell identities for alpha cells, beta cells, delta cells, and PP cells (Figure 2B). scCATCH not only annotated the actual cell type but also identified the potential subtype of cells in each cluster, which are concordantly present among peripheral blood cells in the Gierahn dataset (Figure 2C). For example, scCATCH analysis annotated DCs as

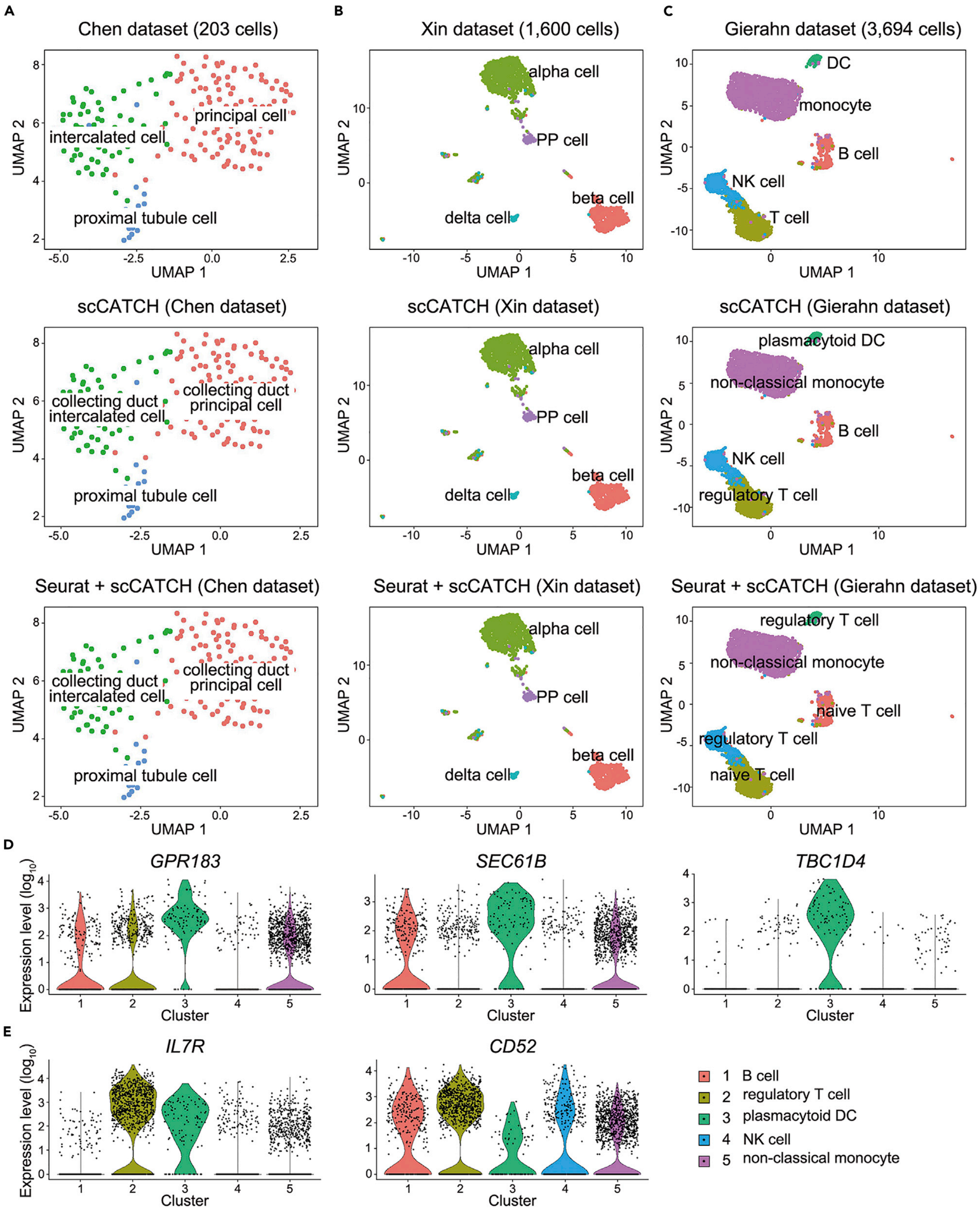

Figure 2. Validation of scCATCH

(A) Validation of scCATCH and identification of cluster marker genes upon Seurat in combination with evidence-based scoring in scCATCH (Seurat + scCATCH) for 203 mouse kidney cells from the Chen dataset.

Figure 2. *Continued*

(B) Validation of scCATCH and Seurat + scCATCH for 1,600 human pancreatic islet cells from the Xin dataset.

(C) Validation of scCATCH and Seurat + scCATCH for 3,694 human peripheral blood cells from Gierahn dataset.

(D) The violin plot of expression levels ($\log_{10}$) for cluster 3 marker genes *GPR183*, *SEC61B*, and *TBC1D4* identified through scCATCH on Gierahn dataset.

(E) The violin plot for the expression levels ($\log_{10}$) of cluster 2 marker genes *IL7R* and *CD52* identified via scCATCH on Gierahn dataset. DC, dendritic cell. NK cell, natural killer cell.

plasmacytoid DCs owing to significant upregulation of plasmacytoid DC marker genes including *GPR183*, *SEC61B*, and *TBC1D4* (Villani et al., 2017) when compared with other clusters (Figure 2D). Moreover, our results marked T cells in the Gierahn dataset as regulatory T cells according to highly expressed *IL7R* and *CD52* in this cluster (Figure 2E). These two genes were proposed as marker genes for regulatory T cells (Haase et al., 2015; Sinha et al., 2018; Wang et al., 2013). In addition, the performance of scCATCH on annotation remains stable with varied number of total cells and clusters.

Potential marker gene selection by scCATCH is indeed interesting. Seurat, a widely used software package for scRNA-seq analysis, was applied herein to identify potential marker genes in the cluster, and the *ES* protocol was determined for annotation. Interestingly, cell types in the Chen and Xin datasets were still accurately labeled, whereas those in the Gierahn dataset were only partially concurrent with the results of scCATCH analysis (Figure 2A), indicating that the *ES* protocol is a robust identifier of cell identity.

Comparison of scCATCH with Other Methods

Cluster potential marker genes markedly contributed to the accuracy of annotation in the cluster-based method. For scCATCH analysis, we carried out paired comparisons to identify differentially expressed genes in only one specific cluster to ensure accuracy in matching the CellMatch database. On the contrary, Seurat uses a one-against-all approach, potentially generating a set of pseudo cluster potential marker genes (highly expressed in at least two clusters). Under this condition, cluster potential marker genes identified through scCATCH analysis usually were a subset of genes determined via Seurat (Figure 3A). However, an increased number of cluster potential marker genes did not benefit cell annotation. Although Seurat accurately annotated cell types common between the Chen and Xin datasets upon scCATCH analysis, Seurat accurately annotated the cell types of only two clusters (40% consistency, Figure 2C) in the Gierahn dataset, namely cluster 2 (T cells) and cluster 5 (monocytes). Apparently, the method of identifying cluster potential marker genes did not differ with a limited number of clusters. On increasing the total number of clusters, scCATCH analysis displayed better performance than Seurat in the identification of actual cluster potential marker genes present in the Gierahn dataset (Figure 2C). For example, *CCL22*, *SWAP70*, and *KLRF1* were identified as cluster potential marker genes via Seurat, with a maximal fold change among the unshared marker genes between Seurat and scCATCH for clusters 1, 3, and 4, respectively. Evidently, *CCL22* and *SWAP70* were upregulated in multiple clusters, whereas *KLRF1* was expressed in some cells in clusters 4 and 5, deterring the differentiation of actual cell types from other clusters (Figure 3B).

Furthermore, validation datasets were used to compare scCATCH with cell-based annotation methods including CellAssign, Garnett, SingleR, scMap, and CHETAH. CellAssign, SingleR, and scMap were able to assign the accurate cell label for most cells, especially pancreatic islet cells in Xin dataset, whereas Garnett and CHETAH barely identified the actual identity of each cell (Figures 3C–3E; Table 1). The consistent rate of Garnett and CHETAH was as low as 0% on the Gierahn dataset, indicating that none of the cells were accurately identified by these two methods.

Owing to cell heterogeneity in the clusters, cell-based strategies could assign multiple cell type labels to one cluster. Our analysis indicated that only 31.6% of proximal tubule cells in cluster 3 of Chen dataset were assigned as proximal tubule cells by SingleR, whereas 36.8% and 31.6% cells in this cluster were assigned as intercalated cells and principle cells, respectively (Figure S1). Besides, for some clusters, most cells' labels (>50%) in the cluster were not consistent with the actual cell type, which presents in all clusters of three validation datasets annotated by Garnett and CHETAH; clusters 1 and 3 of Chen dataset and clusters 3, 4, and 5 of Gierahn dataset annotated by CellAssign; cluster 3 of Chen dataset and most clusters of Gierahn dataset by SingleR; and clusters 3 and 4 of Xin dataset as well as clusters 2 and 4 of Gierahn dataset by scMap (Figures 3C–3E; Table 1). Under this condition, it is hard to assign an accurate cell label to this cluster.

Reference dataset plays a key role in cell type annotation. We next tested the effect of CellMatch on the performance of SingleR and CHETAH. For SingleR, the databases of the Immunological Genome Project

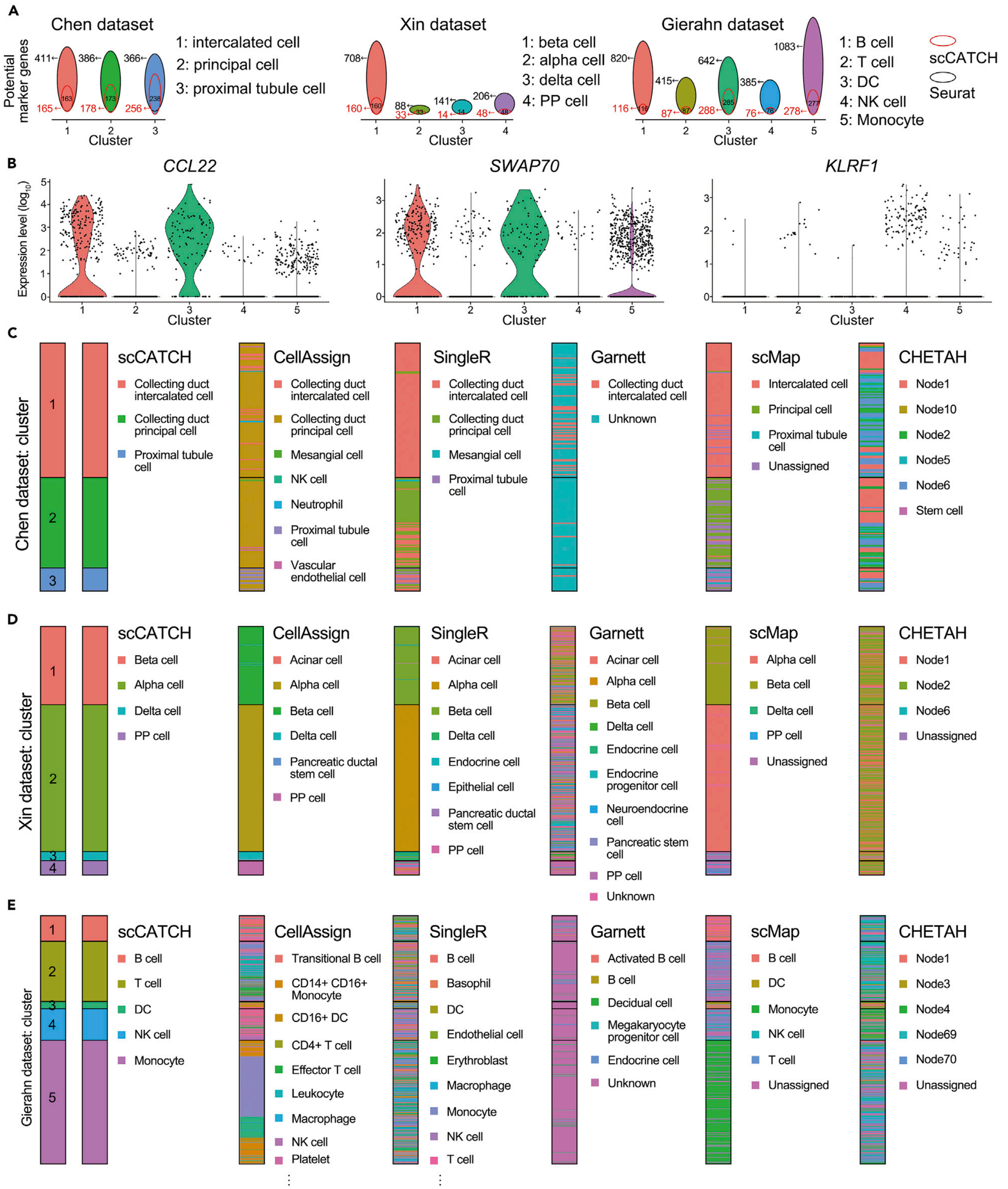

Figure 3. Comparison of scCATCH with Other Methods

(A) Identification of cluster potential marker genes via Seurat (black number beside the circle) and scCATCH (red numbers beside the circle) in three validation datasets in each cluster. The black number inside the circle represents the number of overlapped genes.

(B) The violin plot for the expression levels of cluster 1 marker gene *CCL22*, cluster 3 marker gene *SWAP70*, and cluster 4 marker gene *KLRF1* across 5 clusters identified in the Gierahn dataset via Seurat.

Figure 3. *Continued*

(C) Cell type annotation in the Chen dataset via scCATCH and cell-based annotation including CellAssign, SingleR, Garnett, scMap, and CHETAH.

(D) Cell type annotation in the Xin dataset via scCATCH, CellAssign, SingleR, Garnett, scMap, and CHETAH.

(E) Cell type annotation of the Gierahn dataset. The representative cell types were labeled for CellAssign and SingleR. Nodes 1, 2, 3, 4, 5, 6, 10, 69, and 70 represent intermediate cell types with a low confidence score.

See also Figure S1 and Tables S1 and S2.

(ImmGen) and the mouse RNA-seq were used as the reference list for mouse, whereas the databases HPCA as well as Encode and Blueprint Epigenomics transcriptomes were used as the reference list for human. For CHETAH, a dataset of head and neck was used as the reference. As shown in Tables S1 and S2, using CellMatch as the underlying reference, SingleR performed better on annotating the cells of three validation datasets, especially on non-blood cells, compared with using the ImmGen, mouse RNA-seq, HPCA and Encode, and Blueprint Epigenomics transcriptomes reference lists. Consequently, the consistent rate with CellMatch database by SingleR improved from 0% to 80%–90%. However, CHETAH showed no difference in the consistent rate with CellMatch.

The Performance of scCATCH during Analysis of the scRNA-Seq Dataset

scCATCH was employed to annotate six known scRNA-seq datasets to assess the performance of scCATCH with three recorded in the CellMatch database and three unrecorded. On assessing the internal datasets recorded in the CellMatch database, the Enge dataset (Enge et al., 2017) included 2,281 human pancreatic cells and 6 cell types, namely alpha cells, beta cells, delta cells, acinar cells, ductal cells, and mesenchymal cells, whereas the Wu dataset (Wu et al., 2017) included 20,679 mouse brain cells and 7 cell types including oligodendrocyte precursor cells (OPCs), astrocytes, oligodendrocytes, neurons, microglial cells, endothelial cells, and mural cells. The Lindsey dataset (Plasschaert et al., 2018) included 2,970 human lung cells and 7 cell types including basal cells, brush cells/pulmonary neuroendocrine cells (PNECs), ciliated cells, *FOXN4*+ cells, ionocytes, secretory cells, and *SLC16A7*+ cells. On assessing external datasets that were not recorded in the reference database, the Zheng dataset (Zheng et al., 2017b) included 2,638 human peripheral blood cells and 9 cell types, namely *CD8*+ cells, naive and memory *CD4*+ T cells, *CD14*+ and *FCGR3A*+ monocytes, B cells, NK cells, DCs, and platelets. Moreover, the Zeisel (Zeisel et al., 2015) and Heng (Heng et al., 2019) datasets included 2,915 (7 cell types) and 3,918 (12 cell types) mouse brain cells including neurons, oligodendrocytes, microglia, endothelial cells, mural cells, astrocytes, ependymal cells, neuronal progenitor cells, OPCs, pericytes, and fibroblasts.

In general, scCATCH detected most cell identities and accurately annotated the cluster consistent with the predefined cell type in the literature. Both internal datasets and external datasets displayed an average consistency rate of 83% (Table 2). For 6 cell types in the Enge dataset, scCATCH consistently identified 4 cell types, namely alpha cells, beta cells, delta cells, and acinar cells, and identified clusters 5 and 6 as epithelial cells and acinar cells or beta cells, which were ideally considered as ductal cells and mesenchymal cells (Figure 4A) on the basis of their known marker genes *PROM1* and *THY1*, respectively. However, *PROM1* encodes a pentaspan transmembrane glycoprotein that localizes to membrane protrusions and is expressed on stem cells (Oshima et al., 2007), whereas the protein encoded by *THY1* is expressed in numerous cell types and widely considered as a hematopoietic stem cell marker. As marker genes *EPCAM* (Cheng et al., 2010; Seeberger et al., 2009), *KRT19* (Seeberger et al., 2009), and *CDH1* (Seeberger et al., 2009) were confirmed as marker genes of pancreatic epithelial cells, scCATCH expectedly annotated cluster 5 with epithelial cells instead of ductal cells. Moreover, cluster 6 was identified as acinar or beta cells owing to their equal *ES*s, probably because of the limited cell number in cluster 6, comprising only 54 among 2,811 cells. Regarding the annotation of brain cells in another internal dataset, scCATCH identified all cell types in accordance with the literature, except for mural cells, which were marked as pericytes by scCATCH (Figure 4A). Moreover, pericytes were also considered mural cells, thus indicating 100% accuracy of scCATCH in annotating cell types in the Wu dataset. Among the 7 cell types in the Lindsey dataset, scCATCH accurately identified all cell types consistent with the literature, including basal cells, brush cells/PNECs, ciliated cells, *FOXN4*+ cells, ionocytes, secretory cells, and *SLC16A7*+ cells (Figure 4A). Interestingly, cluster 2, identified as brush cells/PNECs in the literature, was concurrently annotated as brush or neuroendocrine cells owing to their similar *ES*s.

For the Zheng dataset, scCATCH identified the actual cell identities of most clusters along with marker genes of the cluster, such as B cells, *CD8*+ T cells, *CD14*+ and *FCGR3A*+ monocytes, and DCs (Figure 4A). Cluster 1 and 2 were notably labeled as naive and regulatory T cells, which were actually naive *CD4*+ and memory *CD4*+ T cells. However, *CD4* was not upregulated in either cluster 1 or 2 (Figure 4B), thus lacking

Dataset	Cluster	Cell Type	Cell Sum	Consistent Rate					
				scCATCH	Cell Assign	Garnett	SingleR	scMap	CHETAH
Chen	1	Intercalated cell	110	√	13%	28%	98%	90%	0%
	2	Principle cell	74	√	96%	0%	69%	66%	0%
	3	Proximal tubule cell	19	√	47%	0%	32%	21%	0%
	All	NA	203	100%	46%	15%	81%	75%	0%
Xin	1	Beta cell	503	√	98%	47%	95%	94%	0%
	2	Alpha cell	946	√	100%	5%	99%	98%	0%
	3	Delta cell	58	√	93%	48%	67%	9%	0%
	4	PP cell	93	√	100%	45%	72%	16%	0%
	All	NA	1,600	100%	99%	22%	95%	89%	0%
Gierahn	1	B cell	376	√	75%	8%	1%	64%	0%
	2	T cell	903	√	70%	0%	9%	45%	0%
	3	DC	104	√	38%	0%	0%	68%	0%
	4	NK cell	471	√	10%	0%	55%	37%	0%
	5	Monocyte	1,840	√	28%	0%	1%	90%	0%
	All	NA	3,694	100%	41%	0.9%	10%	69%	0%

Table 1. Comparison of scCATCH with Other Methods for Cell Type Annotation

NA, not applicable. √ indicates scCATCH annotates the accurate cell type of the cluster.

The consistent rate of scCATCH and Seurat was determined as the percentage of consistent clusters with the same cell type in each dataset, whereas the consistency of SingleR and CHETAH was determined as the percentage of consistent cell numbers with the same cell type. See also Tables S1 and S2.

evidence regarding clusters 1 and 2 to be annotated on the basis of *CD4* expression. Moreover, the cluster 2 marker gene *IL7R* common between scCATCH and the Zheng dataset was considered a potential marker gene of regulatory T cells (Haase et al., 2015; Wang et al., 2013). Cluster 7 seemed difficult to annotate on the basis of cluster marker genes *GNLY* and *NKG7* of NK cells and cluster marker gene *CD63* of basophils in peripheral blood, whereas cluster 9, containing only 14 cells, expressed both platelet (*PPBP*) and naive T cell (*ACTN1*, *ARHGAP45*, *LDLRAP1*, and *R3HDM4*) marker genes (Zheng et al., 2017a).

Furthermore, two scRNA-seq datasets from mouse brain were selected for scCATCH analysis. At the level of cell type identification, scCATCH accurately annotated the primary cell types including neurons, oligodendrocytes, microglia, endothelial cells, pericytes, astrocytes, and ependymal cells (Figure 4A). Notably, cluster 1 (interneurons), cluster 2 (S1 pyramidal neurons), and cluster 3 (CA1 pyramidal neurons) in the Zeisel dataset were annotated with type IC spiral ganglionic neurons, neurons, and neurons via scCATCH. These may be due to limited number of records on markers for interneurons and pyramidal neurons. Moreover, vascular smooth muscle cells (VSMCs) were annotated with mural cells, concurrent with the fact that mural cells include VMSCs in the Zeisel dataset. For Heng dataset, 9 of 12 cell types annotated via scCATCH were consistent with the literature, whereas 3 other clusters (neural progenitor cells, VSMCs, and brain fibroblasts) were labeled as neuroblasts and type I and type II spiral ganglionic neurons via scCATCH (Figure 4A). scCATCH identified marker genes *Dcx*, *Ccnd2*, *Crmp1*, *Dbn1*, *Dlx2*, *Pfn2*, *Btg1*, *Meis2*, *Stmn2*, and *Dlx6os1* (Luo et al., 2015; Shah et al., 2018; Zhang et al., 2009) for cluster 3, leading to a high possibility as neuroblasts. However, it is difficult to determine the cell type of clusters 5 and 11 since cluster 5 includes marker genes for type I spiral ganglionic neurons such as *Cdc42ep3*, *Mgst3*, *Mob2*, *Nexn*, *Rap1a*, and *Tpm1* for type I spiral ganglionic neurons (Shrestha et al., 2018) and cluster 11 includes marker genes for type II spiral ganglionic neurons like *Adm*, *Bmp7*, *Islr*, *Oat*, *Serpinf1*, and *Wls*. (Shrestha et al., 2018). Regarding their subtypes, numerous marker genes of type I spiral ganglionic

Dataset	Recorded in CellMatch	Species	Tissue	Cell Sum	Cluster Sum	Consistent Rate
Enge	Yes	Human	Pancreas	2,281	6	4/6
Wu	Yes	Mouse	Brain	20,679	7	6/7
Lindsey	Yes	Human	Lung	2,970	7	7/7
Zheng	No	Human	PBMCs	2,638	9	7/9
Zeisel	No	Mouse	Brain	2,915	10	9/10
Heng	No	Mouse	Brain	3,918	12	9/12

Table 2. Evaluation of scCATCH with Internal and External Datasets

PBMCs, peripheral blood mononuclear cells.

neurons were observed in cluster 9 when compared with the original annotation of glutamatergic neurons. The GABAergic neurons in cluster 12 were annotated with type IC spiral ganglionic neurons via scCATCH owing to limited reference data and a limited number of cells.

DISCUSSION

In this study, we developed scCATCH, a cluster-based automatic annotation toolkit for scRNA-seq analysis, which uses a tissue-specific cell taxonomy reference database (CellMatch) and *ES* protocol to annotate cell types. We not only validated the extremely high feasibility of *ES* protocol in scCATCH but also demonstrated the superiority of scCATCH over other methods of identifying marker genes, including Seurat, the cell-based annotation method CellAssign, Garnett, SingleR, scMap, and CHETAH, through three scRNA-seq validation datasets. Moreover, scCATCH was used to assess six other known scRNA-seq datasets wherein scCATCH accurately annotated most cell types.

Thus far, common cell type annotation methods primarily include the cluster-based method by matching single or several representative cluster potential marker genes with known cell markers, which is usually carried out manually; however, such a method tends to require subjective prior knowledge among investigators, and the unstable selection of cluster potential marker genes from the pool ranging from tens to hundreds of cluster potential marker genes results in poor replicability of cell type annotation. Hence, the complete CellMatch reference database and the *ES* protocol were introduced into scCATCH, wherein the *ES* protocol was primarily based on the matched number of supporting studies and validated marker genes. The cell type with the most evidence would be selected as the ultimate annotation. Together with the frequently used Seurat and *ES* protocol, this study indicates the high feasibility of scCATCH for the most annotated cell types concordant with the literature. Compared with manual annotation, the present method prevents manual selection of marker genes and subjective cell type determination. Without the requirement of prior knowledge, scCATCH rapidly, accurately, and reproducibly automatically annotates cell types of clusters from scRNA-seq data.

Recently, some cell-based annotation methods have increasingly emerged to identify the cell type at the single-cell level rather than single-cluster level. CellAssign, Garnett, SingleR, scMap, and CHETAH are known methods in cell-based category that mapped the expression profile of each cell with reference profiles of known cell types. However, for biomedical research, researchers usually are more interested in cell cluster(s) that show different patterns during physiological process, disease development, or drug treatment. This could be the underlying reason for the common workflow of scRNA-seq studies to perform cluster analysis first, followed by annotating cell types using marker genes. Besides, for cell-based methods, the major challenge lies in the determination of cell types on each cluster. Uncertainty may be introduced by cell heterogeneity as shown by our analysis. In addition, for the common cluster-based strategy, an accurate and reproducible toolkit for automatically annotating cells without prior knowledge was unavailable. Hence, we developed scCATCH to help biologists to address the current challenges. Moreover, scCATCH displayed extreme superiority to the cell-based annotation methods, not only upon solid tissue cell type identification but also upon blood cell type identification.

In this study, CellMatch, a comprehensive tissue-specific cell taxonomy reference database till date, was constructed for scCATCH as the underlying data. Application of CellMatch to SingleR and CellAssign resulted in significant improvement in accuracies of cell type annotations, which suggests the great utility of CellMatch as a reference database. Furthermore, as our understanding on cell types continues to be

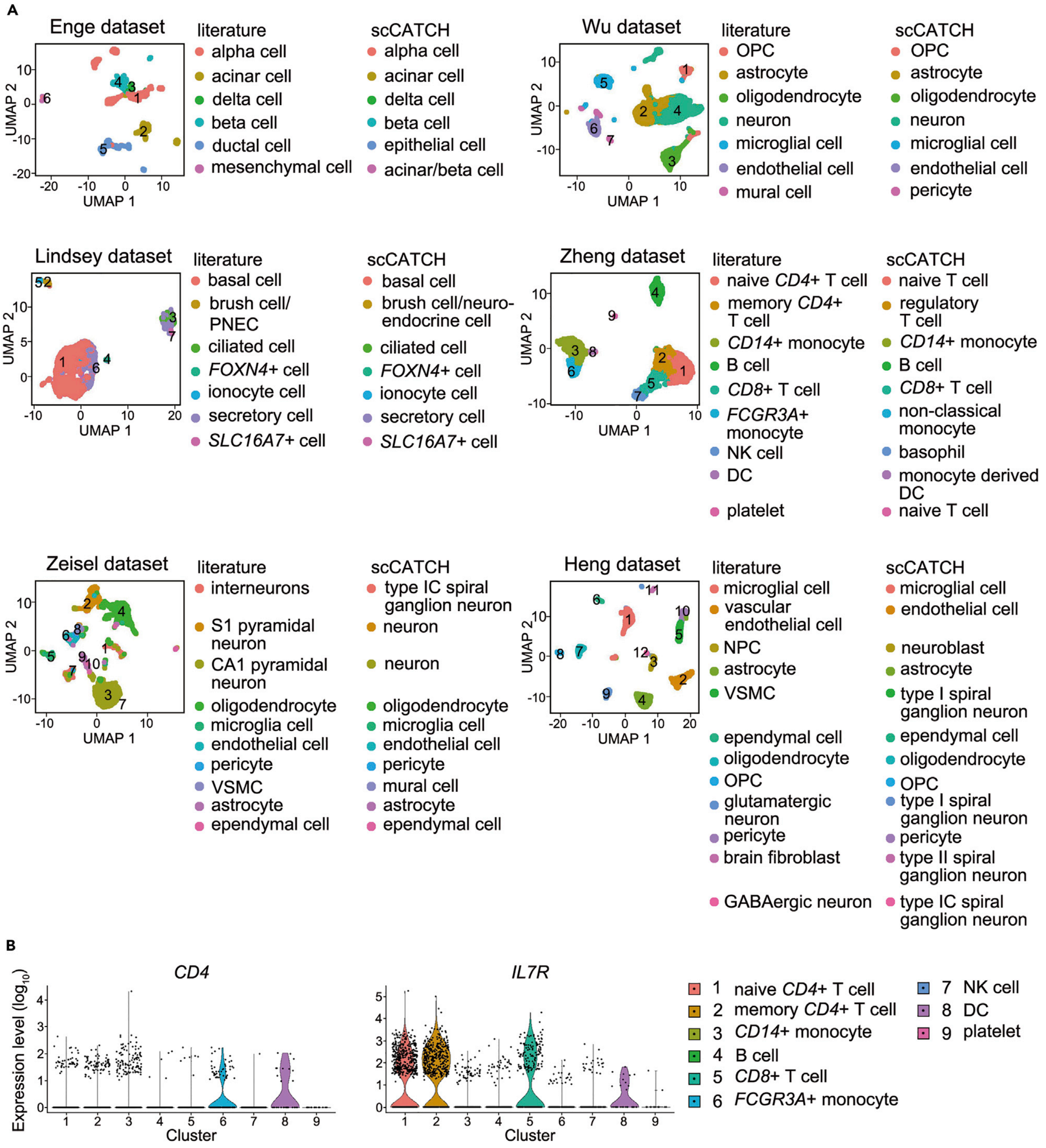

Figure 4. Evaluation of scCATCH

(A) Cell types were annotated via scCATCH in three internal and three external datasets. Cluster numbers are provided with the corresponding cells. Cell types are listed in each cluster. OPC, oligodendrocyte precursor cell; PNEC, pulmonary neuroendocrine cell; DC, dendritic cell; VSMC, vascular smooth muscle cell; NPC, neuronal progenitor cell.

(B) The violin plot for the expression levels ($\log_{10}$) of *CD4* and *IL7R* across 9 cell clusters in the Zheng dataset.

enriched, more marker gene information would be supplemented in this reference and its performance could be further enhanced. In the workflow of scRNA-Seq analysis, clustering is of key importance to the conclusions. For cell type annotation, inadequate clustering analysis also would introduce errors into this process as too many or few cells are both problematic for labeling. It is interesting to evaluate the effects of multiple clustering algorithm on cell type annotations in the future.

In summary, this study describes the development of an automatic and efficient toolkit for the identification of cluster potential marker genes on the basis of *ES* protocol and annotation by constructing a comprehensive tissue-specific cell taxonomy reference database (CellMatch) as the underlying data. The feasibility and availability of scCATCH were systematically validated in different datasets. The present scCATCH analysis would potentially facilitate rapid and accurate identification of actual cell identities without prior knowledge with high replicability. The present results would greatly benefit studies on scRNA-seq data through the elucidation of the cell composition in complex tissues and provide novel insights into mechanisms underlying disease pathogenesis and progression.

Limitations of the Study

The performance of scCATCH majorly depends on the reference database. The limited reference database potentially led to incorrect annotation of cell types via scCATCH.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND CODE AVAILABILITY

The source codes and results are implemented in R and are freely available (https://github.com/ZJUFanLab/scCATCH; https://github.com/ZJUFanLab/scCATCH_performance_comparison). No new data were generated for this study. All data used in this study are publicly available.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100882.

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AUTHOR CONTRIBUTIONS

X.F. conceived and designed the study. X.L. and R.X. collected and analyzed the single-cell RNA-seq data. X.S., J.L., and X.F. implemented the algorithm of scCATCH. X.S., J.L., and N.A. developed the toolkit of scCATCH. All authors wrote the manuscript, read, and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no conflicts of interests.

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Supplemental Information

scCATCH: Automatic Annotation on Cell Types of Clusters from Single-Cell RNA Sequencing Data

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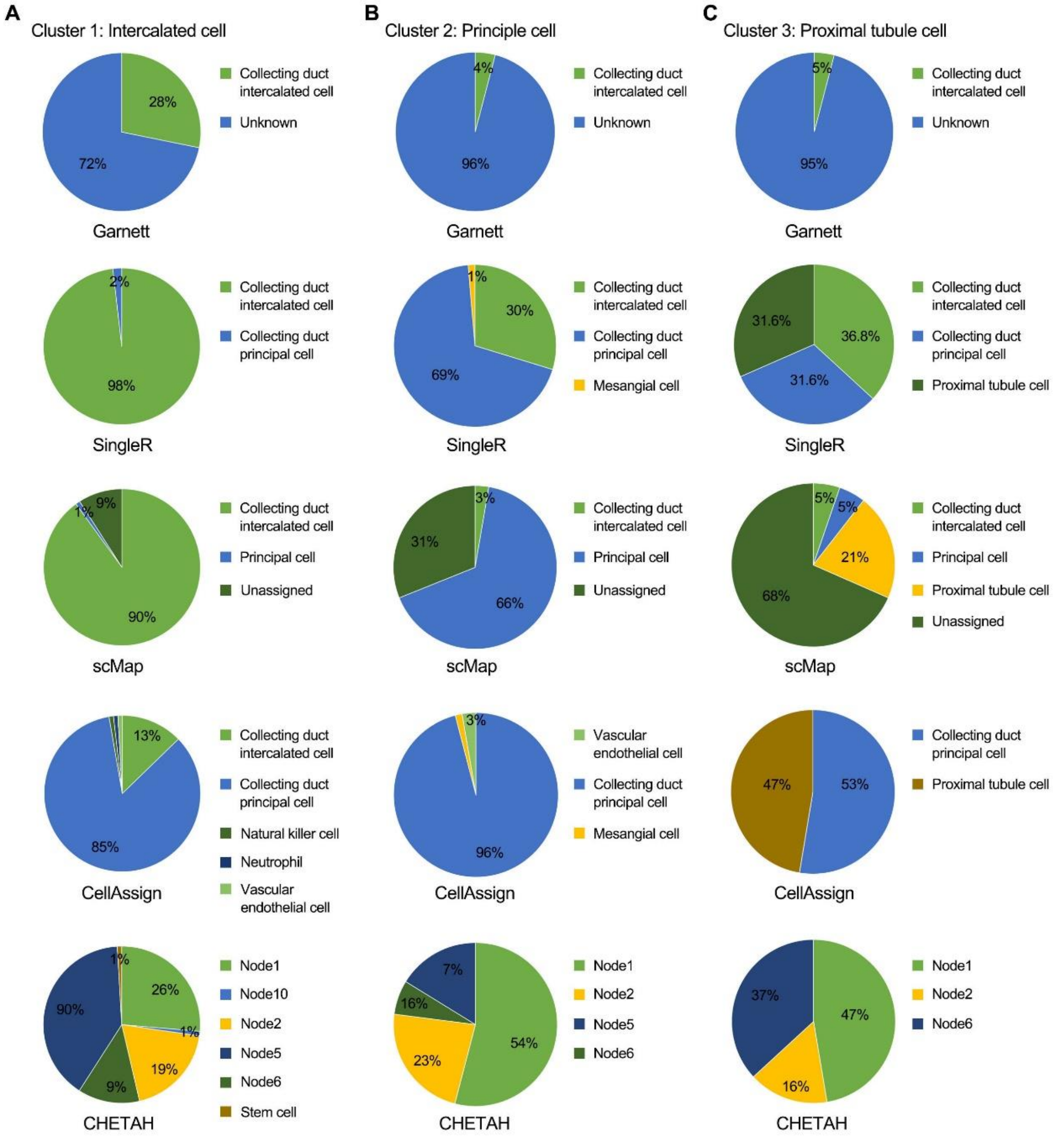

Figure S1.Cell composition in each cluster of Chen datasets, Related to Figure 3 and Table 1. Cell composition in cluster 1 **(A)**, cluster 2 **(B)** and cluster 3 **(C)**, annotated by Garnett, SingleR, scMap, CellAssign and CHETAH.

Table S1. Cell annotation by SingleR and CHETAH with various referred databases on Chen datasets, Related to Figure 3 and Table 1.

Dataset	Cluster	Cell type	Consistent rate				
			SingleR referred database			CHETAH referred database	
			Immgen	Mouse.RNAseq	CellMatch	Headneck	CellMatch
Chen	1	Intercalated cell	0%	0%	98%	NA	0%
	2	Principle cell	0%	0%	69%	NA	0%
	3	Proximal tubule cell	0%	0%	32%	NA	0%
	All	-	0%	0%	81%	NA	0%

Table S2. Cell annotation by SingleR and CHETAH with various referred databases on Xin and Gierahn datasets, Related to Figure 3 and Table 1.

Dataset	Cluster	Cell type	Consistent rate				
			SingleR referred database			CHETAH referred database	
			HPCA	Blueprint.encode	CellMatch	Headneck	CellMatch
Xin	1	Beta cell	0%	0%	95%	0%	0%
	2	Alpha cell	0%	0%	99%	0%	0%
	3	Delta cell	0%	0%	67%	0%	0%
	4	PP cell	0%	0%	72%	0%	0%
	All	-	0%	0%	95%	0%	0%
Gierahn	1	B cell	62%	56%	1%	0%	0%
	2	T cell	90%	95%	9%	20%	0%
	3	DC	74%	88%	0%	4%	0%
	4	NK cell	84%	75%	55%	0%	0%
	5	Monocyte	89%	52%	1%	0%	0%
	All	-	85%	67%	10%	5%	0%

Table S3. The clustering method and initialization platform for all datasets, Related to Table 1 and Table 2.

Dataset	Clustering method	Initialization platform	Compatibility
Chen	Seurat package	C1 Fluidigm system	√
Xin	Seurat package	C1 Fluidigm system	√
Gierahn	Seurat package	Seq-Well	√
Enge	NA	Smart-seq2	√
Wu	Louvain-Jaccard graph clustering	Drop-Seq	√
Lindsey	SPRING	Droplet microfluidics	√
Zheng	Seurat package	10X Genomics	√
Zeisel	BackSPIN	C1 Fluidigm system	√
Heng	K-means clustering	10X Genomics	√

NA, not available. √ represent the compatibility with the pipeline of scCATCH.

Transparent Methods

Datasets

scRNA-seq datasets were retrieved from several high-quality reports and Gene Expression Omnibus (GEO), including human and mouse primary tissues such as peripheral blood, brain, lung, kidney, and pancreas, wherein unannotated cells were excluded. The Zheng dataset (2,700 peripheral blood mononuclear cells [PBMCs]) was directly downloaded from Satija Lab (https://satijalab.org/seurat/). Validation datasets included the Chen, Xin, and Gierahn datasets, wherein cell types were experimentally validated via FACS or in situ hybridization and IHC. Test datasets included three internal datasets of Enge, Wu, and Lindsey and three external datasets of Zheng, Zeisel, and Heng, wherein cell types were annotated using known marker genes.

Construction of the CellMatch reference database

Human and mouse cell markers from CellMarker (http://biocc.hrbmu.edu.cn/CellMarker), MCA (https://figshare.com/articles/MCA_DGE_Data/5435866), CancerSEA (http://biocc.hrbmu.edu.cn/CancerSEA), and the CD Marker Handbook (http://static.bdbiosciences.com/documents/cd_marker_handbook.pdf) were retrieved.

For CellMarker database, cell markers derived from undefined tissue were excluded in this study and only cell markers with at least one supporting article were included. After integrating cell markers from the same tissue of origin, it led to 45,090 records (28,636 for human and 16,454 for mouse) involving 175 tissue types, 635 cell types, 20,213 cell marker genes and 2,085 references.

For MCA database, raw counts and the meta information of cell types were downloaded and processed. For each tissue, cells with the same annotation were merged and cell marker genes for each cell type were identified with FindAllMarkers of Seurat by using Normalized data via LogNormalize, in which the percentage of expressed cells was set to 75%, P value from Wilcoxon Rank-Sum (WRS) test to 0.01, and log10 fold change to 0.5. After the integration of marker gene from same tissue origin, it led to a total of 4,014 records related with 31 tissue types, 139 cell types, and 1,486 cell marker genes.

For CancerSEA database, cell marker genes were curated from cancer related single-cell studies, wherein studies sourced from tissue were included. A total of 1,196 records were obtained from 11 references related with 6 tissue types, 27 cell types as well as 997 cell marker genes.

For CD Marker Handbook database, human and mouse key cell markers were collected as blood cell markers. A total of 54 records were obtained from CD Marker Handbook database related with 15 cell types and 50 cell marker genes.

Cell marker gene symbols and gene IDs were revised in accordance with NCBI gene data (https://www.ncbi.nlm.nih.gov/gene/) updated on July 1, 2019, wherein unmatched genes were removed from the CellMatch. Repeated records were combined, and cell types and subtypes were extracted from the names of annotated cells in accordance with histological origin, expression of specific markers or degrees of differentiation. To ensure the accuracy of CellMatch, manual confirmation were performed via independently examining the marker genes and reference by three reviewers. Lastly, cell marker genes curated from CellMarker, MCA, CancerSEA and CD Marker Handbook database were integrated to establish species-specific and tissue-specific reference database CellMatch, which includes 49,635 records (29,836 for human and 19,799 for mouse) involving 184 tissue types, 353 cell types and related 686 subtypes, 20,792 cell marker genes and 2,097 references.

Data pre-processing

All scRNA-seq data were processed using R (version 3.6.1). For Zheng datasets, the raw count was processed in accordance with the pipeline of the Satija Lab tutorial, using Seurat 3.0, wherein cells with unique feature counts of >2,500 or <200 and >5% mitochondrial counts were filtered out. For other datasets, all cells in the datasets were included in the filtered matrices and the meta information of cell clusters and cell types for all cells were obtained from the literature. Cells with same annotation were merged into the same cluster, and duplicated genes were combined through summation of raw counts for each cell. All datasets were then saved as the CellDataSet class prepared for running scCATCH and other methods.

Data preparation for scCATCH

All datasets were transferred as Seurat objects from CellDataSet objects by extracting raw count and meta information of cell clusters and cell types. Then the raw counts were normalized via the global-scaling normalization method *LogNormalize*. Principal component analyses (PCA) were performed followed by uniform manifold approximation and projection (UMAP) analysis for dimensional reduction and visualization. All datasets were stored as Seurat objects prepared for running scCATCH.

Identification of cluster potential marker genes with scCATCH

For clusters i and j among n clusters ($i \neq j$, i & $j \leq n$), $G_{i,j}$ was defined as the gene set in which every gene's average expression in cluster i is significantly greater than that in cluster j with the percentage of expressed cells (≥25%), using WRS test (P<0.05) and a $\log_{10}$ fold change of ≥0.25. For each cluster i, the cluster potential marker gene set M_i was obtained using the following equation:

$$M_i = G_{i,1} \cap G_{i,2} \cap G_{i,\ldots} \cap G_{i,j}$$

Cluster annotating process with scCATCH using cluster potential marker genes

Evidence-based scoring (*ES*) protocol in scCATCH involved two steps. The first step was to determine the cell type, and the second step was to determine the subtype of the corresponding cell type. For each cluster i, the cluster marker gene set M_i was matched with species-specific (human or mouse) and tissue-specific (blood, brain, kidney, etc.) cell markers from CellMatch database on the basis of revised gene symbols. c_i was considered as the matched unique cell type candidates. For each candidate k among c_i, the ES_k was determined as follows:

$$ES_k = \sqrt{\frac{l_k}{l_k + 1} \times \frac{g_k}{g_k + 1}} \quad (1)$$

In equation (1), l_k represents the unique number of related studies, while g_k is the number of associated cell marker genes, referring to the intersection of set M_i and the cell markers in

CellMatch database. Candidate *k* with the maximal ES_k was determined as the cell type for cluster *i*. Furthermore, s_i was considered the matched unique subtypes belonging to candidate *k*. For each subtype *m* among s_i, the $ES_{k,m}$ was determined as follows:

$$ES_{k,m} = \sqrt{\frac{l_{k,m}}{l_{k,m}+1} \times \frac{g_{k,m}}{g_{k,m}+1}} \quad (2)$$

In equation (2), $l_{k,m}$ represents the unique number of related evidence/reference while $g_{k,m}$ is the number of associated cell marker genes. Subtype *m* with the maximal $ES_{k,m}$ (> 0.5) was determined as the cell subtype for cluster *i*.

For scCATCH annotation, the mouse kidney cell markers was selected from CellMatch database for Chen dataset. The human pancreas and pancreatic islet cell markers were used for Xin and Enge datasets. The human blood, peripheral blood, and bone marrow cell markers were picked for Gierahn and Zheng datasets; mouse brain cell markers for Wu, Zeisel, and Heng datasets; human lung cell markers for Lindsey dataset.

Annotation with cluster potential marker genes identified by Seurat and scCATCH

For Seurat, cluster potential marker genes of three validation datasets were identified with *FindAllMarkers*, wherein Normalized data via “LogNormalize” were processed to determine the positive cluster potential marker genes with default parameters with the percentage of expressed cells (>10%), using WRS test (P<0.01) and a $\log_{10}$ fold change >0.25. For scCATCH, cluster potential marker genes of three validation datasets were identified with *findmarkergenes*, wherein Normalized data via *LogNormalize* were processed to determine the positive cluster potential marker genes with default parameters with the percentage of expressed cells (≥25%), using WRS test (P<0.05) and a $\log_{10}$ fold change ≥0.25. Both cluster potential marker genes generated from Seurat and scCATCH were used to annotate the cell types of three validation datasets via the function of *scCATCH* on the basis of *ES*s. As previously described, the tissue type for the Chen dataset, Xin dataset and Gierahn dataset was set to kidney, pancreas and pancreatic islet and blood, peripheral blood, and bone marrow, respectively, when annotating.

Performance comparison on the validation datasets with various methods

For CellAssign, three validation datasets were first transformed as SingleCellExperiment objects from CellDataSet object by extracting raw count and meta information of cell types. CellMatch database was then used as reference while all other parameter in CellAssign were kept as default (learning rate, 0.01).

For Garnett, the marker genes of mouse kidney cells, human pancreas and pancreatic islet cells, and human blood, peripheral blood and bone marrow cells from CellMatch database were first extracted to train corresponding classifiers of three validation datasets. The parameter of the number of unknown type cells was set as an outgroup during classification with a value of 50. Then the trained classifiers were used to classify the cells of Chen, Xin and Gierahn datasets.

For SingleR, three validation datasets were first transformed as a SingleR object from CellDataSet object by extracting raw count and meta information of cell types. To annotate Chen dataset by SingleR, the default databases of the Immunological Genome Project (ImmGen) and the mouse RNA-seq were used as the reference list, wherein a new reference list generated from CellMatch database by extracting the marker genes of mouse kidney cells was incorporated. To annotate Xin and Gierahn dataset by SingleR, the default databases of HPCA as well as Encode and Blueprint Epigenomics transcriptomes were used as the reference list, wherein a new reference list generated from CellMatch database by extracting the marker genes of human pancreas and pancreatic islet cells was incorporated for Xin dataset, and another new reference list by extracting the marker genes of human blood, peripheral blood and bone marrow cells was incorporated for Gierahn dataset.

For scMap, three validation datasets were transformed as SingleCellExperiment objects from CellDataSet object by extracting raw count and meta information of cell types. The three validation datasets were normalized with $\log_2$ raw counts and duplicated genes were removed from the normalized matrices. The individual cells in each dataset were used as the reference to projects cells of the corresponding dataset by scMap-cell.

For CHETAH, three validation datasets were transformed as SingleCellExperiment objects from CellDataSet object by extracting raw count and meta information of cell types. To annotate Chen, Xin and Gierahn datasets by CHETAH, the default reference of head and neck were used and three new references were created and added by extracting the corresponding marker genes from CellMatch database, as previously described in SingleR.

Consistent rate evaluation

Consistent rate for scCATCH was defined as the percentage of consistent clusters annotated with the same cell type as in the literature, while consistent rate for CellAssign, Garnett, SingleR, scMap and CHETAH was defined as the percentage of consistent cells with the same cell type, as in the literature.

Code and data availability

The source code of scCATCH is implemented in R and is freely available at https://github.com/ZJUFanLab/scCATCH. The source code and results of performance comparison on the detail of the process among scCATCH, CellAssign, Garnett, SingleR, scMap and CHETAH, and CellMatch database are implemented in R and is freely available at https://github.com/ZJUFanLab/scCATCH_performance_comparison. All data are accessible in GEO with the following accession codes: (a) for Chen dataset, the accession code is GSE99701; (b) Xin dataset, GSE81608; (c) Gierahn dataset, GSM2486333; (d) Enge dataset, GSE81547; (f) Wu dataset, GSE103976; (g) Lindsey dataset, GSE102580; (h) Zeisel dataset, GSE60361; (i) Heng dataset, GSE125708.