Engineering 16 (2022) 187–197

Contents lists available at ScienceDirect

Engineering

journal homepage: www.elsevier.com/locate/eng

Research
Neuroimmunology—Review

Applications of CyTOF in Brain Immune Component Studies

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ARTICLE INFO

Article history:
Received 5 November 2020
Revised 16 March 2021
Accepted 3 June 2021
Available online 28 August 2021

Keywords:
CyTOF
Brain
Immune components
Neuroinflammation

ABSTRACT

The brain is the most heterogeneous and complex tissue in the body. Previous studies have shown that immune cells are essential functional components in both healthy and pathological brains. Cytometry by the time of flight (CyTOF) is a high-dimensional single-cell detection technology that allows measurements of up to 100 cell markers with a small number of samples. This technique enables the identification and characterization of various cell types at the single-cell level under steady-state and diseased brain conditions. This review outlines three major advantages of the CyTOF technique compared with the traditional flow cytometry approach. We also discuss CyTOF applications in brain immune cell component research in both healthy and pathological brains.

1. Background

The brain is the most complex organ in the body; it is composed of various types of cells and functions as the "headquarters" of the body. Apart from neurons, astrocytes, oligodendrocytes, and the glia cells, the brain has its own residential immune cells: the microglia. For decades, it has been commonly accepted that immune cells—including both brain residential and circulating immune cells—are essential parts of the brain under both healthy and diseased conditions [1]. For example, during the fetal-to-adult transition in the healthy brain, microglia require cluster of differentiation 69^{+} (CD69^{+}) CD4 T cells to complete this process in both mice and humans [2]. Under pathological conditions, Hughes and Appel [3] have shown that microglia dysfunction attenuates the activity of neurons and oligodendrocytes by myelination and that myelin abnormalities are hallmarks of neurological diseases. In neuroinflammatory and neurodegenerative diseases, including Parkinson's disease (PD), multiple sclerosis (MS), Alzheimer's disease (AD), and Huntington's disease (HD), the migration of peripheral immune cells is considered to be the beginning of pathology. Therefore, identifying and applying phenotypic markers is a key to distinguishing the various immune cell types in the brain.

Ter119 is a distinctive marker for separating the brain residential microglia from the infiltrating monocyte-derived macrophages (MoDMs)—two cell types with similar morphology and behavior under pathological conditions [4]. Functional analysis is also an essential part of brain immune component studies and is not limited to identifying the brain immune cell populations. Studies have shown that the same immune cells may play different roles at different stages of the disease. For example, in ischemic stroke, MoDMs enhance the pro-inflammatory responses in the acute phase but switch to an anti-inflammatory role during the chronic phase [5].

Therefore, to meet the increasing demands of both phenotype and functional studies of brain immune components, a more detailed, precise, and high-dimensional technique is required. However, the traditional immunology technique, fluorescence-activated cell sorting (FACS), has limitations in meeting such information-intensive needs. Cytometry by the time of flight (CyTOF) is a high-dimensional, high-throughput, single-cell analysis technique with metal-labeled antibodies, which can detect up to 100 parameters simultaneously. In this review, we summarize the application of CyTOF in brain-related studies to discuss the basics, advantages, and disadvantages of CyTOF, and to

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https://doi.org/10.1016/j.eng.2021.06.022

evaluate its potential use in the clinical diagnosis of brain-related diseases.

2. CyTOF is a powerful technique to systematically analyze cell types

2.1. History of mass cytometry

CyTOF, which is also known as mass cytometry, is an advanced technology for detecting metal-conjugated cell markers, both on the cell surface and inside the cell. It was first discovered by Bandura et al. [6] at the University of Toronto. Bandura et al. adopted the principle of inductively coupled plasma mass spectrometry, which is used to detect elemental materials in fluids. Elemental composition materials (e.g., lanthanides) are extremely low in abundance or almost absent in biological systems. Bandura et al. utilized metals, instead of fluorochromes, as a detector. This technique is commonly used for general pathology diagnosis. In 2011, Bendall et al. [7] from Stanford University first adopted this technique in the field of immunology. Bendall et al. analyzed 34 parameters using single-cell suspensions from human bone marrow to reveal the differential immune cells and drug responses in human hematopoietic disease. This technology has been applied in immunology research since then, and the number of parameters in the detection panel has expanded significantly. This new approach overcomes the fluorophore overlap problems that occur when using traditional FACS [6,8,9]. A comparison of CyTOF and FACS is summarized in Table 1 [10].

2.2. The principle of CyTOF

CyTOF is mainly based on the time-of-flight (TOF) mass spectrometer principle. TOF is extremely accurate in determining the mass-to-charge ratio of an ion. During the acquisition process, all ions are accelerated through an electric field with known strength, and the time taken for these ions to reach the detector over a known distance is measured. The heavier the ion, the longer it takes to get to the detector. To perform TOF, cells are vaporized and only the isotopes are kept. The CyTOF technique was developed based on the TOF principle, using antibodies conjugated to various ions. Ions are collected to determine the expression of the conjugated antibodies, allowing the immune cell types defined by the antibodies to be identified.

2.3. Three major advantages of CyTOF

CyTOF has three major advantages. First, CyTOF has significantly increased detection capacity. When using this approach, the barcode technique is applied to investigate up to 20 samples in a single mix. As the CyTOF procedure requires each sample to be barcoded before the staining step, all 20 samples undergo identical staining, washing, and permeabilization steps as a combined single multiplexed sample. This procedure can minimize variations among samples to generate more consistent and accurate results. In addition to the harmonization of sample preparation, this deconvolution of the barcode procedure can largely prevent cell doublets from forming between differently barcoded samples, thereby reducing intrasample doublets [11]. In addition, the number of probes used in one reaction is significantly increased from 18 (for FACS) to 40–50 panels (for CyTOF) [6], and some studies have shown that a CyTOF panel can include as many as 100 channels [12]. As various immune cell types are identified and distinguished based on their cell surface markers, CyTOF permits the simultaneous detection of many more markers using the same sample. In rare instances, up to ten cell surface markers may be required to accurately define one cell type. With the CyTOF technique, the detection capability expands by 4–5 times, and a greater number of markers can be applied in each sample, further providing the opportunity for an in-depth investigation [13]. For example, in human peripheral blood mononuclear cells (PBMCs), the natural killer (NK) cell subsets are defined as ($CD45^+CD3^-CD19^-CD20^-CD14^-HLA^-DR^-CD38^+CD16^+$) and the myeloid dendritic cell (DC) subsets are defined as ($CD45^+CD3^-CD19^-CD20^-CD14^-HLA^-DR^+CD11c^+CD123^-$) [13]. These cell markers, along with live/dead stains, may occupy almost all of the available panels when the traditional FACS method is used, which limits the further study of cell functional markers using the same sample. NK cells have been used as a treatment for malignancy due to their antitumor activities, which may increase significantly after stimulation with various cytokines. Vendrame et al. [14] studied the NK cell repertoire after cytokine stimulation using the CyTOF approach. In addition to typical cell surface phenotype markers and intracellular functional cytokine markers, they included the NK cell receptors (NKG2A, NKG2C, and NKG2D), natural cytotoxicity receptors (NKp30, NKp44, and NKp46), and killer cell immunoglobulin-like receptors (KIR2DL1, KIR2DL2/L3/S2, KIR2DL3, KIR2DL5, KIR3DL1, KIR3DL1/S1, and KIR3DL2). These markers were used to cluster the sub-NK population based on their differentially expressed marker patterns in response to various cytokine stimulations [14]. Similarly, Hansmann et al. [15] discovered a novel memory B cell population expressing both memory ($CD27^+$) and naïve ($CD24^{low}CD38^+$) phenotypes using CyTOF. All of these new discoveries took advantage of the increased detection capability of CyTOF.

Second, CyTOF dramatically decreases spectral overlap and background noise. As Mazza et al. [16] have shown, when using multichannel fluorescence flow cytometry methods, the background fluorescence and spreading errors must be considered, as they are the major contributors to increase the variability and decrease accuracy. This is because, instead of being conjugated to fluorochromes, the antibodies used in the CyTOF technique are conjugated to elemental composition materials, such as lanthanides. These elemental compositions have little or no expression in biological systems; thus, the background of CyTOF is generally close to an undetectable level. In addition, the detection overlap among heavy metal isotopes is generally limited to less than 2% [17], whereas conventional FACS has a spectral overlap of 5%–100%. Furthermore, traditional FACS uses fluorochromes as tags. These molecules have a wide range of molecular weights, from the small protein fluorescein isothiocyanate (FITC) to the larger molecule allophycocyanin (APC). Furthermore, each fluorochrome has its own excitation and emission [18]. In CyTOF, the metal tags all have similar chemical characteristics, as they belong to the lanthanide family, thus increasing the overall sensitivities [18]. Takahashi et al. [19] showed that, when designing the CyTOF panel, it is necessary to consider using 89Y, 113In, or 115In with high-intensity markers, as these three lanthanide elements are relatively less sensitive. Nevertheless, the chemical similarity of

Table 1
Comparison between CyTOF and FACS.

Feature	CyTOF	FACS
Detectable probe	Metal probe	Fluorescent probe
Compensation	No	Yes
Maximum detectable probes	Up to 50 (~100)	< 20
Mixed sample	Yes (barcode)	Not common[a]
Protection from light	No	Yes
Fix before stain	Yes	No
Permeabilization	Yes	Only when staining intracellular marker
Data analysis	Relatively difficult	Relatively easy
Major data analysis software	Cytobank	Flowjo

[a] There are few barcode FACS protocols (e.g., T cell tetramer stain [10]).

the lanthanide elements enables CyTOF to provide much more accurate data in comparison with the traditional FACS approach.

Third, CyTOF can provide reliable quantification with low numbers of cell input. Gadalla et al. [20] provided a protocol that enables reliable quantification by means of CyTOF down to a low number of input human cells—an approach that is particularly important when cell numbers are limited. Their data show that CyTOF can faithfully recapitulate FACS data in both PBMCs and tumor tissues, thereby providing reliable staining of more than 35 parameters for high-dimensional analyses in the analysis of cancer clinical trials. Scientists from Yale University showed that this high-dimensional technique can reveal cellular samples from an extremely limited sample size, such as 1–2 mL of blood or small biopsy pieces [13]. To determine the minimal cell number detectable using CyTOF, Yao et al. [13] analyzed various immune cell types, including T cells, B cells, NK cells, monocytes, myeloid DCs (mDCs), and plasmacytoid DCs (pDCs), after serial dilutions of human peripheral blood. Their results showed that CyTOF revealed the same percentage of lymphocytes from PBMCs when the total number of cells dropped from 1×10^6 to 1×10^4 mL^{-1} through serial dilutions [13].

3. Application of CyTOF in healthy and pathological brains

Traditional flow cytometry has a limited capacity to detect multiple fluorescence parameters due to the overlapping of fluorochrome spectra. With its high-dimensional characteristics, CyTOF is capable of examining the brain's immune environment to show the immune cell landscape both in a naïve brain (i.e., healthy conditions) and under pathological conditions, including neuroinflammation and neurodegenerative disease (Fig. 1).

3.1. CyTOF application in the immune microenvironment of healthy brains

The central nervous system (CNS), which includes the brain, spinal cord, optic nerves, and retina, does not have a classic lymphatic drainage system. Thus, the brain is traditionally considered to be an immune-privileged organ [21]. However, an increasing amount of data indicate that immune cells are not only required to prevent infections [22,23], but also play important roles in maintaining a healthy brain.

3.1.1. Immune cells play key roles in healthy brain functions

Under healthy conditions, immune cells play a key role in maintaining brain function. Brynskikh et al. [24] showed that immune cells directly participate in brain learning. To be specific, immune-deficient mice or those with adaptive immunity depletion have dramatically impaired learning outcomes compared with wild-type mice [24]. Furthermore, Ziv et al. [25] showed that $CD4^+$ T cells contribute to the maintenance of neurogenesis and spatial learning in adults. In addition, studies have shown that the proper functioning of adaptive immunity is important for maintaining mental activity and coping with conditions that may lead to cognitive deficits. For example, Kipnis et al. [26] showed that T cell-depleted mature mice developed cognitive deficits and behavioral abnormalities, but then recovered after T cell restoration. It is notable that the immune compartment of the brain comprises not only the residential immune cells—that is, microglia—but also infiltrating immune cells. For example, Shechter et al. [27] reported that immune cells can enter immune-privileged sites in the steady state through the fenestrated vasculature envelope, which acts as an immune-skewing gate. Furthermore, Louveau et al. [22] showed that there is constant immune surveillance in the meningeal compartments. These data suggest that immune cells are present in the brain under naïve conditions.

3.1.2. The CyTOF technique reveals the immune cell components in healthy brains

It is technically challenging to evaluate the roles of immune cells under healthy steady-state due to the extremely low number of immune cells in healthy brains. This is particularly evident when the traditional FACS approach is used. Recent advances in next-generation sequencing (NGS) have provided the gene expression profiles of residential myeloid cells in various parts of the brain [28]; however, the sequencing data only show gene expression at the transcriptional level, not at the protein level. In 2017, Korin et al. [29] used the high-dimensional CyTOF technique to systematically review the immune population and characterize the brain immune compartment in naïve mice. Using 44 cell surface markers, they first described the immune cell subsets in the naïve brain, including $CD8^+$ T cells, B cells, NK cells, and DCs [29,30]. Remarkably, by comparing the immune cell composition in the brain with that in the peripheral blood, CD44 was identified as a marker of brain-infiltrated leukocytes [29]. In 2018, Mrdjen et al. [31] further proved that there are substantial amounts of leukocytes within the normal CNS, including brain residential microglia and macrophage-like cells, classic DCs (cDCs), pDCs, B cells, NK cells, and natural killer T (NKT) cells.

3.2. CyTOF application in the immune microenvironment of pathological brains

Challenges by internal or external stimuli evoke inflammation in the brain, in what is known as neuroinflammation. During neuroinflammation, the landscape of leukocytes changes dramatically; however, the commonly used image-dependent immunohistochemistry or traditional FACS have a limited capacity to identify various types of immune cells using a small number of samples. CyTOF, a high-dimensional single-cell detection technique, can comprehensively reveal the leukocyte map and the entire immune landscape during neurodegeneration and neuroinflammation.

3.2.1. CyTOF application in neurodegeneration disease research

Based on a previously created proteome atlas of immune populations [6,32], Becher et al. [32] used CyTOF to extensively characterize various leukocyte subsets using neurodegeneration mouse models. They identified the immune landscape during the steady-state CNS. Combined with functional assays, these scholars identified an immune cell population located in the region of the CNS, in contrast to the parenchyma—namely, the CNS border-associated macrophages (BAMs), which can be distinguished by CD38 and major histocompatibility complex class II (MHCII) [32]. They also identified microglia, DCs, and monocytes as separate populations in naïve brains, aided by extensive cell surface and protein phenotype markers. These data were later corroborated by both Mrdjen et al. [31] and Korin et al. [29], who revealed additional immune cell populations by comparing data from the steady-state and pathological conditions. Active microglia were found to express higher levels of CD11c and CD14, accompanied by unregulated CD86 and CD44 and the down-regulation of programmed death ligand-1 (PD-L1) expression [31]. A slight increase was also found in the expression of MHCII in microglia, while the microglia homeostatic checkpoint markers, chemokine C–X3–C motif receptor 1 (CX3CR1), Mer tyrosine kinase (MerTK), and sialic acid-binding immunoglobulin-like lectin (Siglec)-H, decreased [31]. Using the mouse autoimmune encephalomyelitis (EAE) model and the MS mouse model, Mrdjen et al. [31] reported that the majority of the infiltrated cells were monocyte-derived cells, followed by T cells. Phenotypically, the resident microglia are the

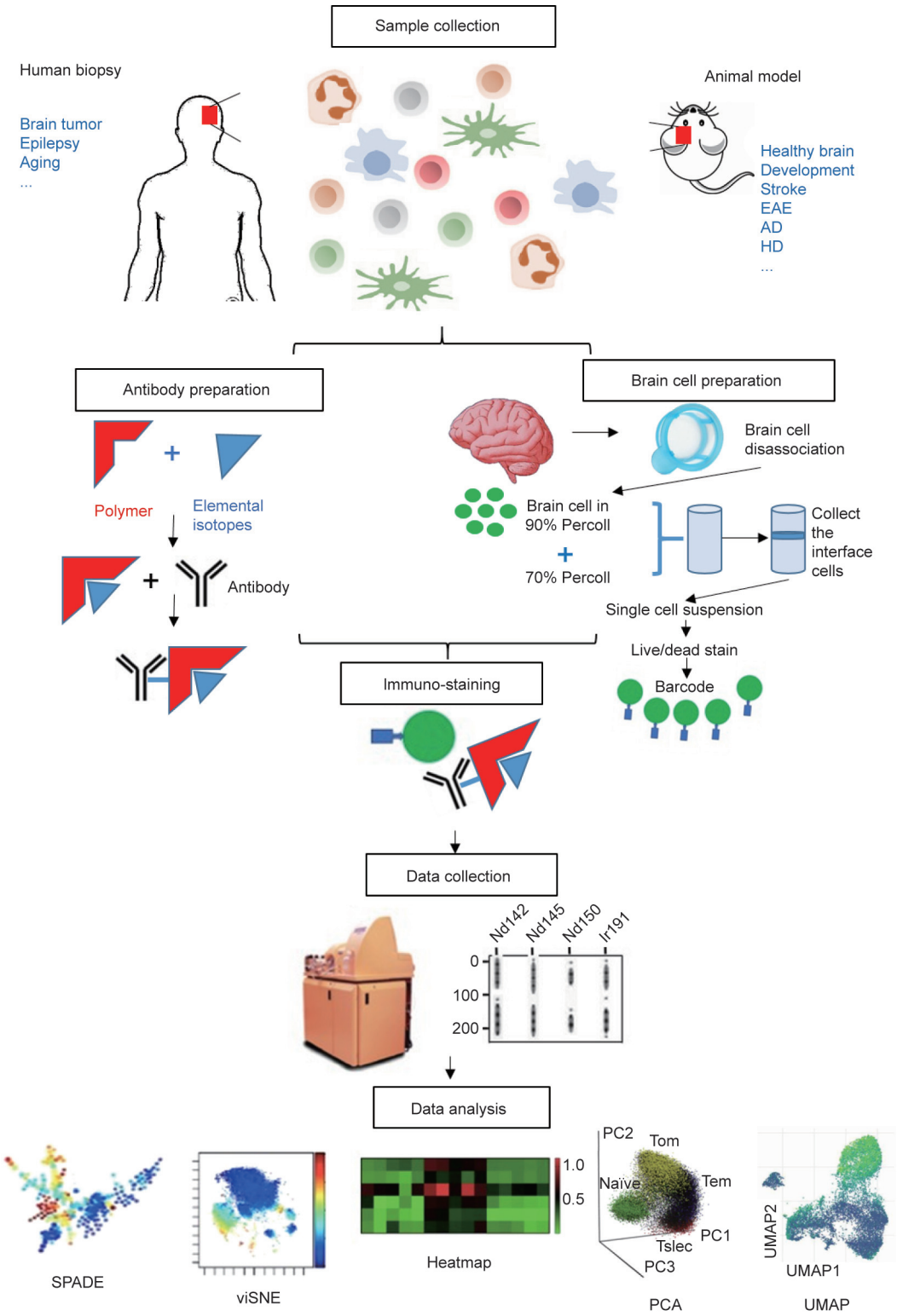

Fig. 1. Overview of the CyTOF experimental procedure. Samples are collected from human biopsies or animal models. For antibody preparation, a conjugated antibody with a unique heavy metal isotope is used. To prepare the brain single-cell suspension, these single cells are first stained as live or dead and then marked with a specific barcode. Single cells are stained with ion-conjugated antibodies. The acquired labeled single cells are assayed using a CyTOF machine. Data analysis can be performed using Cytobank and represented using (but not limited to) spanning-tree progression analysis of density-normalized events (SPADE), visualization of high-dimensional single-cell data based on the *t*-distributed stochastic neighbor embedding (viSNE), heatmaps, principal component analysis (PCA), or uniform manifold approximation and projection (UMAP). EAE: encephalomyelitis; AD: Alzheimer's disease; HD: Huntington's disease; Nd: neodymium; Ir: iridium. Nd142, Nd145, Nd150, and Ir191 stand for various CyTOF channels; Tom, Tem, and Tslec stand for various cell types identified by CyTOF.

most active cells, as demonstrated by the activation of phenotypic markers [31]. Similar to the immune responses in AD, the expressions of the microglia homeostatic checkpoint markers CX3CR1, MerTK, and Siglec-H were reduced in the EAE model, while the expression of CD86, PD-L1, and CD44 was increased. In contrast to the EAE model, the expression of CD14 was decreased, while the level of Sca-1 was increased in the AD mouse model [31].

In another investigation, Ajami et al. [33] discovered three novel $CD11b^+$ brain myeloid cell populations using CyTOF in an EAE mouse model, and named the three cell populations A, B, and C, respectively. These myeloid cell populations are only found in the brain and are absent in the peripheral blood. All three cell populations expressed the typical CD45, CD11b, CD317, and CD39 markers. Populations B and C also expressed MHCII and CD86, while population A was MHCII and CD86 negative. MHCII and CD86 have previously been identified as myeloid cell activation markers [34], suggesting that populations B and C—but not population A—were activated microglia populations [33]. With signaling phenotype markers, including transcriptional factors and signaling factors, such as phosphorylated signal transducer and activator of transcription (STAT) transcription factors (pSTAT1, pSTAT3, pSTAT5), phosphorylated cyclic adenosine monophosphate (cAMP) response element-binding protein (pDREB), phosphorylated kinase (pMAPKAPK2), and nuclear factor-κB (NF-κB), Ajami et al. [33] showed that populations B and C highly expressed these signaling proteins while population A did not. These results confirmed that populations B and C were indeed active populations with highly expressed functional markers. To further study the function of these three cell populations, R6/2 mice in an HD model and mice overexpressing mutant human superoxide dismutase as a model of amyotrophic lateral sclerosis (ALS) [35] were used [33]. In the HD mouse model, all three cell populations existed before and during the disease progression. Moreover, the frequencies of populations A and B increased, while those of population C remained at a low level over the course of HD progression. The increases in populations of microglia in the ALS mouse model were consistent with previous reports that microglia numbers were increased in the HD model [35]. In summary, this study systematically studied the activity status of immune cells, particularly that of the three different groups of microglia (A, B, and C). Their phenotypes showed significant changes under steady-state in comparison with under neurodegenerative disease conditions, providing in-depth information on brain immune responses.

Furthermore, Willis et al. [36] identified a unique $CD8^+$ T cell population using CyTOF in an MS mouse model. Guilliams et al. [37] presented an effective way to identify DC populations across various mouse tissues, including the lung, spleen, intestines, kidney, liver, and brain, using an unsupervised mass cytometry approach. Their data also revealed the heterogeneity of DCs across both human and mouse tissues, which had not been fully identified or defined previously using conventional cytometry [37].

3.2.2. CyTOF is a powerful technique to systemically study the whole picture of neuroinflammation induced by ischemic stroke

Stroke is one of the leading causes of death in humans and results in 6.2 million deaths annually worldwide [38]. Ischemic stroke—the most common acute cerebrovascular disease—accounts for approximately 80% of stroke cases worldwide [39]. Ischemia injury induces neurological deficits including microvascular failure, damage to the brain-blood barrier (BBB), oxidative stress, and brain edema [40]. Dead neurons release damage-associated molecular patterns (DAMPs), which further cause neuroinflammation in the ischemic hemisphere [41]. This focal brain inflammation aggravates the secondary brain damage and induces subsequent brain inflammation [42]. After more than 20 years of study, it is now commonly accepted that immune responses play a key role in both the tissue damage and the healing process after ischemic stroke [43].

Previous studies have shown that various immune cells participate in ischemia-induced brain inflammation, such as brain residential microglia [44], infiltrating T cells [45-49], monocytes [5,50], NK cells [51], and neutrophils [52]. For example, to explore the functions of different T cell subsets, severe combined immunodeficient (SCID) mice (immune-deficient animal mice with a B6 genetic background), CD8 T cell-deficient mice (B6.129S2-*Mapk9*^{tm1Flv}/J), and CD4 T cell-impaired mice (B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J) were subjected to middle cerebral artery occlusion (MCAO) surgery. Two days later, the wild-type mice had significantly larger infarctions than the $CD8^-$ and $CD4^-$ mice, suggesting that T cell subsets play a role in infarction sizes and stroke outcome [49]. Furthermore, Gu et al. [49] performed the same surgery using T helper 1 (Th1)-impared mice (B6.129S2-*Mapk9*^{tm1Flv}/J), Th2-impaired mice (C57BL/6-*Il4*^{tm1Nnt}/J), and regulatory T cell (Treg)-impaired mice (B6.129X1-*Ebi3*^{tm1Rsb}/J), and showed that the Th1-impired mice had reduced brain infarction, while Th2-impired mice had aggravated brain injury. These findings suggest that Th1 CD4 T cells have pro-inflammatory functions, while Th2 CD4 T cells have anti-inflammatory functions.

In addition to T cells, peripheral infiltrating leukocytes, such as monocytes, NK cells, and neutrophils, play important roles. For example, the C–C motif chemokine receptor 2 (CCR2) is responsible for the migration of monocytes into the brain. Fang et al. [5] showed that *Ccr2*-knockout (KO) mice had a smaller infarction size and lower mortality than wild-type mice in the acute phase after MCAO. Gan et al. [51] showed that the NK cells accumulate after ischemia via neuron-derived chemokine C–X3–C motif ligand 1 (CX3CL1). Comparing the infarction size of *Rag2*-KO mice (lacking T, NKT, and B cells) and *Rag2* and γc double-KO mice (lacking T, NKT, B, and NK cells), Gan et al. [51] showed that the double-KO mice (which were devoid of NK cells) had a smaller infraction size, suggesting that NK cells also contribute to cerebral infarction and are independent of T and B cells. Furthermore, neutrophils have been found to be involved in stroke-induced neuroinflammation and to determine stroke outcomes [52]. *Myosin1f* is an immune cell migration-relevant gene that is highly expressed in neutrophils [53]. Our research showed that the *Myosin1f*-KO mice had lower neurological deficiency and a smaller infarction size than wild-type mice, suggesting the role of neutrophils in the brain immune response [52].

However, due to their limited channel numbers, traditional FACS techniques cannot be used to integrate the information related to these immune cell changes using the same samples, or to provide a complete picture of stroke-induced neuroinflammation [46]. CyTOF is undoubtedly a better choice to systemically reveal the immune response map. Based on previous FACS channels, we designed a metal-conjugated CyTOF panel to characterize the immune components in the ischemic brain, peripheral blood, spleen, and bone marrow using a mouse model at multiple time points after stroke. We quantified the number of immune cells in these organs from days 1 to 14 after MCAO. Using CyTOF, we quantified the lymphocytes, macrophages/microglia, and DCs from the same ischemic brain hemisphere sample, thereby delimiting acquiring and processing errors during sampling. To better understand immune cell interactions across tissues, we applied *R* programing for the first time to analyze the network among the immune cells from the brain, peripheral blood, spleen, and bone marrow, with the aim of analyzing the links among these immune cells across various organs and different timeframes. Our data showed that the immune cells across the whole body had "linked" together by one day after an ischemic stroke, suggesting that stroke should be considered a whole-body immune response rather than solely a brain disease. This result enables us to obtain a better overview of the immune response after stroke in

multi-organ aspects across time and provides a new idea for stroke clinical treatment [54].

3.2.3. CyTOF application in brain tumor research

Primary and metastatic brain tumors are the third leading cause of cancer among young adults aged 20–39. Current standard treatments, such as surgery, chemotherapy, and radiotherapy, still have many limitations, and the median survival range is only 5–7 years [55,56]. In addition, the highly immunosuppressive tumor microenvironment (TME) allows only a selective group of patients to benefit from immunotherapy. Moreover, a clinical trial of the programmed death-1 (PD-1) blockade (NCT02017717) did not show a survival benefit in recurrent glioblastoma (GBM) [57,58]. As the most heterogeneous tissue, the brain TME not only contains the brain's unique residential immune cells, microglia, but also includes various immune cells from the periphery. Thus, defining the immune signature of a brain tumor helps in understanding the interactions between tumor cells and immune cells, which could help in predicting immunotherapy outcomes [59].

To identify tumor-specific invading leukocytes, Becher et al. [32] employed a total of two CyTOF panels and 74 parameters to comprehensively evaluate myeloid cells and lineage immune cells using 38 *ex vivo* surgical gliomas, brain metastases (BrMs), and non-tumor epilepsy specimens. Their results showed that gliomas and BrMs have distinct TMEs. Tumor-associated macrophages (TAMs) are the dominant immune cells in gliomas, while tumor-infiltrating lymphocytes (TILs) are the major immune cells in the brain. Kiss et al. [60] showed that the TME defined the TAM's plasticity and polarization. Using the CyTOF technique, Friebal et al. [55] showed that the TAM was driven by a specific type of tumor. For example, in GBM, $CD206^+$ peripheral MoDMs promoted the tumor suppression environment, particularly in World Health Organization grade II and III tumors. Using CyTOF, Fu et al. [61] confirmed that, in both initial and recurrent GBM, the glioma-associated microglia/macrophages (GAMs) are the dominant immune cells and contribute to the immune-suppressive characteristics. In contrast to the brain, where the TAMs accumulate, the number of myeloid-derived cells decreases in patients with a brain tumor in the periphery. Moreover, in 2018, Alban et al. [62] analyzed 259 GBM patient blood samples using CyTOF in order to investigate the immune system changes. Their data showed that the myeloid-derived suppressor cells (MDSCs) decreased over time in newly diagnosed patients with glioma. In addition, patients with GBM have a longer survival time, and those with low-grade glioma have decreased MDSCs in the periphery [62]. When Alban et al. [62] compared blood to determine changes in the immune system among tumor types and over the progression of disease, their data showed an increased cell number of non-immunosuppressive conventional Tregs in the blood of patients with GBM. Using comparative CyTOF analysis, Khalsa et al. [63] showed that, in a GBM mouse model, the inert tumor had numerous exhausted CD8 T cells and resident macrophages, while having fewer eosinophils and $Siglec\text{-}F^+$ macrophages. The phenotypes of tumor-infiltrating immune cells (TIICs) in patients with GBM differ significantly from those of the blood lymphocytes of these patients [63].

The CyTOF technique has also been applied to diffuse astrocytoma (DA) and oligodendroglioma (OG), in addition to GBM. Fu et al. [64] analyzed the immune compartments in ten DA and four OG clinical samples with 33 markers. Their data showed that tumor-associated microglia and macrophages presented higher immunosuppressive characteristics in DA than in OG. In addition, the $PD\text{-}1^+$ $CD8^+$ T cells, T cell immunoglobulin domain and mucin domain-3 $(TIM\text{-}3)^+$ $CD4^+$ T cells, and Tregs were all increased in DA, which likely promoted the immune-suppressive microenvironment. It is notable that published data using CyTOF consistently show that both brain residential microglia and peripheral infiltrated macrophages are the predominant immune components in various brain tumors. Therefore, understanding TAM functions would shed light on brain TME research.

3.2.4. CyTOF application in brain epilepsy research

It is estimated that more than 10.5 million children worldwide may have active epilepsy [65]. Previous studies have shown that epilepsy-induced neuroinflammation plays a key role in etiopathology and convulsive disorders [66]. Owens et al. [67] detected the brain-infiltrating lymphocytes (BILs) from ten pediatric epilepsy patients using 20 antibody markers and analyzed them using CyTOF. Their data showed that activated T cells, including CD4, CD8, and γδ T cells, were present in all cases, indicating that an autoimmune response had already occurred in those young patients with severe seizures. Taking advantage of the high-throughput characteristics of CyTOF, they further showed that the Tregs are almost exclusively in the BILs, while active effector memory populations of CD4 T cells ($CD45RO^+$, HLA^-DR^+, and $CD69^+$) were found in all of the BIL factions. Furthermore, these cells also express C–X–C chemokine receptor 3 (CXCR3) and CCR5, suggesting that the resected epileptogenic brain area released chemokines to attract immune cells [67].

4. CyTOF application in microglia research

Microglia account for up to 10% of adult CNS cells. During the embryonic period, the heterogeneity of microglia reaches its highest point, while this heterogeneity gradually decreases in the neonate, juvenile, and adult stages [68]. As the brain's residential immune cells, microglia play an essential role in brain development, homeostasis, neuroinflammation, neurodegenerative disease, and psychiatric diseases [68,69]. Under healthy conditions, microglia functionally support the development of neurons, prune the synapses, and scavenge dead cells [70]. Under diseased conditions, microglia respond to the altered environment and become active, while their heterogeneity dramatically increases [68]. Compared with other immune cell types, microglia are the dominant immune cell type in pathological brains [71]. All of their highly diverse functions make microglia one of the most heterogeneous cell types in the brain.

Based on the highly heterogeneous characteristics of microglia, a single-cell-level detection technique is a preferred approach. Using single-cell sequencing, Sankowski et al. [71] and Masuda et al. [72] studied the spatial and temporal heterogeneity of mouse and human microglia. Their data showed that in microglia, different genes are differentially regulated during the development and disease stages. Their results showed abnormal RNA expression of microglia in MS [72] and in human glioma [71]. With the recent emergence of the CyTOF technique, this technique reveals additional altered markers that can be used to provide information in relevant pathways [68]. More specifically, using a mouse model, Mrdjen et al. [31] and Ajami et al. [33] studied an experimental autoimmune EAE model and an ALS model, and reported that microglia during the disease stage show dramatic changes, in terms of both the cell surface and functional markers. Moreover, CD44 can be used as a marker to distinguish brain residential cells from those that from peripheral blood. To understand the heterogeneity of human microglia, Böttcher et al. [73] applied CyTOF and detected the expression of 57 markers in human microglia from five different brain regions. Their CyTOF data showed that the higher expression of markers is closely associated with microglial activation, such as the up-regulation of CD68, CD86, CD45, and CX3CR1, which were found to be upregulated during the disease stage. Furthermore, these markers are space dependent,

as they are mainly expressed in the thalamus in comparison with other brain regions [73]. In 2019, Sankowski et al. [71] combined both single-cell sequencing and CyTOF techniques and showed that glioma-associated microglia have a disease-associated signature. Using gene ontology enrichment analysis, the most dramatic changes were shown to be closely related to the inflammatory responses (including leukocyte cell–cell adhesion, responses to interferon (IFN)-γ, and phagocytic capabilities) and to responses to oxidative stress [71]. Moreover, their data pointed out that the metabolism of microglia changes in human glioma; to be specific, apolipoprotein E (APOE), which mainly plays a role in binding lipoproteins or lipid complexes in plasma or interstitial fluids to specific cell surface receptors [74]. The expression of APOE in microglia was also reported to have functions in neurodegenerative diseases, particularly in terms of regulating microglia phenotypes [75]. Sankowski et al. [71] showed that APOE was also unregulated in glioma-associated microglia, which further suggests that there might be a close relationship between lipid metabolism and activation in brain-disease-associated microglia.

5. SynTOF: CyTOF application in brain synapse research

The application of SynTOF, which is the use of CyTOF in brain synapse research, is particularly helpful for understanding the composition of cell neuron synapses in the naïve brain. Neuron synapses play a key role in the transmission of signals; however, it is challenging to study biomarker expressions among various cells due to their complexity and heterogeneity. Gajera et al. [76] were the first to adapt CyTOF to analyze human synaptosomes, the enriched neuron terminals from the brain, and named this method "SynTOF." They stained single cells from the collected synapses with a specific neuronal marker panel for synaptosomes. This marker panel included phenotypic markers specific to brain cell types (e.g., CD11b, CD56, CD298, glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP)) and synapses (e.g., CD47, dopamine transporter (DAT), norepinephrine transporter (NET), and glutamate transporters). The panel also included several functional markers, such as microtubule-associated protein 1 light chain 3 beta (LC3B), which is involved in autophagy, 3-nitrotyrosine for oxidative damage, and K48-ubiquitin for proteasome degradation [76,77]. SynTOF data were then visualized and analyzed using both spanning-tree progression analysis of density-normalized events (SPADE) [78] and visualization of high-dimensional single-cell data based on the *t*-distributed stochastic neighbor embedding (viSNE) [79].

6. Conclusion and perspective

It is challenging to investigate cell markers and their phenotypes at the single-cell level in complex heterogeneous tissues, particularly in the brain. Peripheral leukocytes migrate into the brain via the BBB during neuroinflammation, resulting in an even more complex brain microenvironment. The high-dimensional CyTOF technique, with its significantly increased number of staining channels using a smaller amount of samples, makes it possible to "see" the whole picture composed of various immune cells, tumor cells, or synapses at the single-cell level. It also provides an opportunity to understand in depth how the phenotypic markers of a cell are linked to its functions and cell signaling. More importantly, CyTOF makes it possible to overview the networks of complicated cell subtypes and their relationships (Fig. 2).

6.1. Limitations of CyTOF

CyTOF has demonstrated predominant advantages over traditional FACS and other approaches. However, a few shortcomings are difficult to overcome. First, the cells are destroyed during the acquisition stage. Therefore, FACS analysis and cell sorting may still be required after using CyTOF in order to identify the cell of interest. Second, the cost of antibody preparation and labeling remains excessively high, preventing CyTOF's widespread application. Third, although technically there is no signal interference between mass channels, isotopic impurities may cause contaminations among different channels. Thus, an optimal panel design is

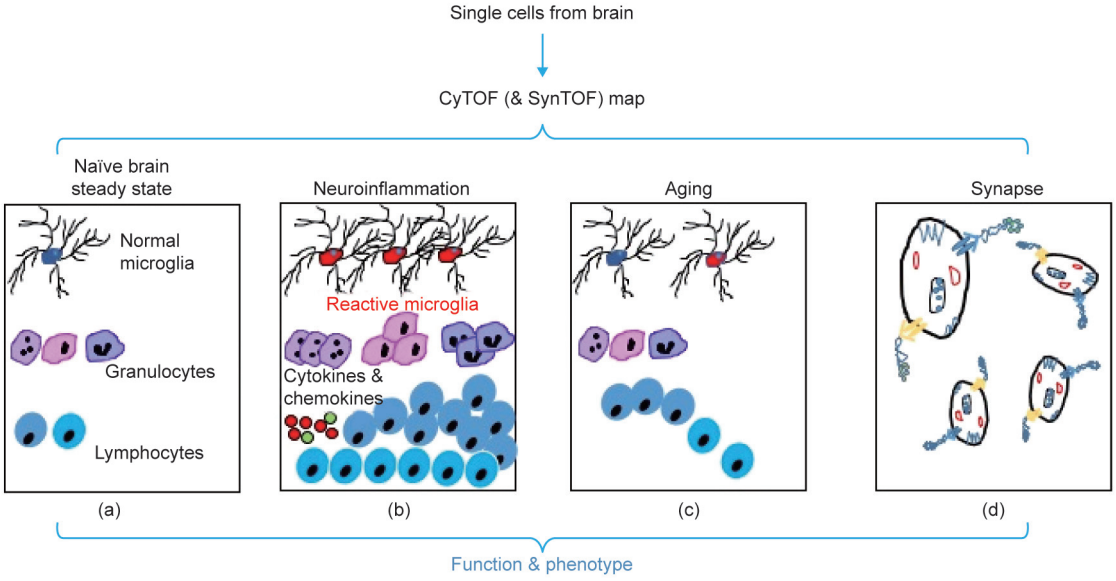

Fig. 2. Mass cytometry, or CyTOF, identifies brain immune cells and synapses under both steady-state and pathological conditions. (a) In a healthy naïve brain, the CyTOF technique can detect a small number of immune cells. (b) Under neuroinflammation conditions (including neurodegenerative diseases, stroke, and brain tumor), the CyTOF technique can identify the phenotype, functional, and activation markers of various immune cells. (c) In an aged brain, this method can reveal the immune cell status. Cells with branches represent microglia (blue represents the naïve condition and red represents the pathological condition); purple and pink cells with multiple nuclei represent granulocytes, dark/light blue cells represent lymphocytes, red solid circles represent chemokines, and green solid circles represent cytokines. (d) SynTOF can reveal the types of synapses using cell type markers (blue lines) or functional markers (yellow/red lines).

required in order to maximize the signal and minimize interference. Takahashi et al. [19] used PBMCs as experimental material to optimize the CyTOF panel design. Their results showed that the signal strength, intensity of the marker, sensitivity of the channel, and dynamics of signal interference are essential factors that should be considered [19]. More specifically, a relatively intense marker, such as CD45, should be paired with ^{89}Y, ^{113}In, or ^{115}In, as these isotopes are relatively less sensitive [19]. Like traditional FACS, the major function of CyTOF is to detect and analyze the immune cell population and its subtypes via cell surface and intracellular marker expression. In 2013, Newell et al. [80] developed a technology that combines tetramer staining with CyTOF to facilitate T cell epitope mapping and characterization. This method can screen up to 109 peptide-MHC tetramers using a human blood sample. If this technology could be used in brain-relevant studies, it would help us to determine the possible epitopes in the aging process and in brain-relevant diseases. This method might be particularly useful in brain infection to predict which epitopes will be recognized by T cells in individuals [81]. Finally, CyTOF has been mainly used to study various immune components; however, it is still unknown how this technique will advance research in other fields of study [76]. It is encouraging to see some initial applications of CyTOF to study cell populations and their functions in various human diseases, such as septic shock [82], rheumatic disease, and melanoma [83], from the bedside.

6.2. The new trend of combining of CyTOF and single-cell sequencing

The combination of single-cell sequencing and CyTOF has become a notable trend for rapidly and accurately investigating gene information, expression, and function at the single-cell level. More importantly, these two techniques are complementary, as single-cell sequencing provides genetic information at the transcriptional level, while CyTOF shows gene expression levels at the translational level. Since 2014, there have been some in-depth brain studies that have applied single-cell sequencing in their research. Patel et al. [84] applied single-cell RNA sequencing to reveal intratumoral heterogeneity in primary GBM. Pollen et al. [85] showed an activated signaling pathway in the heterogenetic developing cerebral cortex using low-coverage single-cell messenger RNA (mRNA) sequencing. As sequencing has rapidly upgraded, this technique can now not only reveal sequence information, but also show gene modifications, such as methylation, in both DNA and RNA [86].

Similar to single-cell sequencing, CyTOF is a high-throughput and high-dimensional technique. However, in contrast to the sequencing technique, CyTOF can reveal information at the protein expression level. Therefore, combining these two techniques can provide a more comprehensive map at the single-cell level. This type of combination study has been applied to lacrimal gland regeneration [87] and human PBMC heterogeneity studies [88]. As the brain is the most heterogeneous tissue, this combination will become a promising way to reveal a more comprehensive picture of the brain, including genetic and protein information changes under various biological conditions or at different developmental stages. For example, Sankowski et al. [71] used a mouse model to utilize single-cell-based immune phenotyping by CyTOF and single-cell RNA sequencing to profile the heterogeneity of microglia and other CNS-associated macrophages (CAMs) in mice. The features of single-cell sequencing and CyTOF are listed in Table 2 [32,78,79,89–96].

New methods have also emerged, such as the cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq). Using oligonucleotide-labeled antibodies, CITE-seq can simultaneously measure single-cell transcriptome and surface proteins by sequencing [97]. In 2017, Peterson et al. [98] developed a combination method to measure gene and protein expression levels with DNA-labeled antibodies using droplet microfluidics at the single-cell level, which they termed "RNA expression and protein sequencing assay" (REAP-seq). Their data showed that this method could detect more than 20 000 genes in a single workflow and quantified proteins with 82 barcoded antibodies. Both these methods can provide high-throughput cell information at the transcriptional and translational levels. Thus, these two methods may provide future approaches to detect information at both the RNA and protein levels for brain immune component studies at the single-cell level.

6.3. The new trend of combining CyTOF and imaging mass cytometry

Recently, new techniques have been developed to expand the detection dimensions of CyTOF from studying single cells in suspensions to analyzing paraffin-embedded tissue sections. Imaging mass cytometry (IMC) was first performed in 2014 [99]. In this method, laser ablation is applied to analyze the plumes of particles using inert gas. The images can be reconstructed from the tissue sections scanner by means of IMC, making this method comparable to microscopy [100].

Similar to IMC, multiplexed ion beam imaging by the time of flight (MIBI-TOF) is a new trending technology that can be used to study how the phenotype of individual cells relates to the function of the multicellular structures by quantifying the spatial distribution of multiple proteins across large regions of intact tissue at a subcellular resolution [101]. This method can achieve multiplexed imaging with high resolution and sensitivity. These two techniques have been commonly used to analyze human cancer tissue biopsies. They can not only reveal the TME of patients with oral squamous cell carcinoma [102] and breast cancer [103], but also be used to study TILs in cutaneous melanoma [104]. The spatial information reveals where the information was obtained and how various cells construct the tissue. Thus, the combination of

Table 2
Features of CyTOF and single-cell sequencing.

Feature	CyTOF	Single-cell sequencing
Detectable level	Translational	Transcriptional
Modification	Yes (post-translational modification)	Yes (methylation)
Require single-cell suspension preparation	Yes	Yes
Cell isolation	On the CyTOF machine	Sorting [89]; manodroplet dilution [90]; microwell [90]
Mixed sample	Yes (barcode)	Yes (barcode)
Fix	Yes	No
Applied cell types	Limited cell types (e.g., immune cells, synapse)	Generally applied to various cell types
Data analysis	Relatively difficult	Relatively difficult
Major data analysis software/programs	Cytobank (not limited to viSNE [79,91], SPADE [78], PCA [92], UMAP [93], Citrus [94], Scaffold [95], X-shift [96], DEnsVM [32])	Program *R* (other programming/algorithms [89])

either IMC or MIBI-TOF approaches with CyTOF could yield more high-dimensional data in future tissue-sample studies [105].

6.4. Emergence of advanced flow cell cytometry: Cytec® Aurora

Cytec® Aurora is an advanced flow cell cytometer with five lasers and three scattering channels, which can detect up to 64 fluorescence channels. The main limitation of traditional FACS is the overlap among various fluorochromes; Cytec® Aurora overcomes this problem by utilizing the differences in full emission spectra signatures to resolve the combinations. Park et al. [106] applied a 40-color panel to detect the immune phenotype of major cell subsets in human peripheral blood. To avoid the need for fixation permeabilization, they only included cell surface markers. Sahir et al. [107] developed a 43-color panel to characterize T cell subsets, B cells, NK cells, monocytes, and DCs from PBMCs. They also pointed out that this technique helps interpret the immune components when the specimen quantity is low [107]. Riggs et al. [108] applied Cytec® Aurora to study the macrophages in acute murine gammaherpesvirus 68 (MHV68) infection, thereby suggesting its potential for application in disease-related studies. Requiring the same staining protocol as traditional FACS, which is relatively easy, Cytec® Aurora has huge potential to apply in the neuroinflammation field.

6.5. Analyzing and visualizing mass cytometry data

As the CyTOF technique significantly increases the detectable numbers of phenotype and functional characters at the single-cell level, it generates more data and requires the development of data analysis approaches [109]. The process of CyTOF data acquisition and normalization requires at least six steps before analysis, including calibration, cell detection, normalization, de-barcoding, rescaling, and randomization [110]. For beginners to analyze CyTOF data, three major algorithms can be run via the Cytobank platform (i.e., viSNE, SPADE, and Citrus) [111], which require less computational background and provide commentary visualization approaches [109,111]. More specifically, viSNE can convey subtle variations and rare populations, while SPADE provides a tree model that simplifies the cell number and structure. When using SPADE, it is necessary to judiciously select the nodes presented on the tree, as an arbitrary misrepresentation of the cell population information might result from choosing the default node number setting [78]. Kimball et al. [109] have pointed out that X-shift and PhenoGraph are better at presenting the quantity of the cell clusters and have the potential to extract a larger amount of data. Furthermore, both principal component analysis (PCA) and *t*-stochastic neighborhood embedding (*t*-SNE) are used for dimensionality reduction. While PCA reduces the high-dimensional space by linearly transforming the data, *t*-SNE is linear. Uniform manifold approximation and projection (UMAP) is a new and better technique in comparison with *t*-SNE, as it increases the analysis speed and provides better preservation of the global structure of the data; thus, UMAP has been used in recent immune component studies [93,112].

In summary, CyTOF's costs, longer process procedures, and more complex data visualization and quantitation remain challenging for CyTOF users. Nevertheless, this new high-dimensional technique has revolutionized investigation at the single-cell level. Limited information exists regarding the brain; nevertheless, as a high-throughput, high-dimensional single-cell analyzing technology, CyTOF has facilitated the in-depth investigation of single cells in multiple aspects. Armed with this powerful tool, scientists can visualize and analyze a single cell in a deeper, more integrative, and more precise manner, making it possible to accurately and efficiently dissect the basic characteristics of cells that have never been observed before.

Acknowledgments

This work was supported by the Beijing Natural Science Foundation (7214269), the National Natural Science Foundation of China (82001248), and the Peking University Third Hospital Talent Program C (BYSYZD2019047).

Compliance with ethics guidelines

Yan Wang, Baohui Xu, and Lixiang Xue declare that they have no conflict of interest or financial conflicts to disclose.

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