# Cytometry by Time-of-Flight (CyTOF) and its Application in Brain Immunology



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## Abstract
The brain is a nervous tissue's mass at the anterior end of a living organism and it integrates sensory information and directs motor responses. It is also a learning center for higher vertebrates. The human brain weighs approximately 3 pounds and consists of billions of cells called neurons. Previous studies indicate that immune cells are essential functional components in the healthy and pathological brain. Cytometry by time-of-flight (CyTOF) is a high-dimensional single-cell detection technology that allows the measurement of up to 100 cell markers with a small number of samples. This technique aims to measure the physical and chemical characteristics of particles individually, and thus animal, plant, and microbial cells can be often examined with this method. This technique makes it possible to detect different types of single cells under stable and diseased brain conditions; hence, the present study used a review approach to investigate this technique and its various applications in brain immunology and discussed the ability of this technique and its empirical results.

**Keywords**: Cytometry, Brain, Brain immunology, Cell diagnosis
**DOI Number: 10.48047/NQ.2023.21.3.NQ33025** **NeuroQuantology2023;21(3): 240-258**

## 1. Introduction
Immune cells consist of a diverse and dynamic cell population that is responsible for a wide range of immune activities. They act harmoniously through communication with other immune and non-immune cells. Understanding this complex immune network requires widespread characterization of its cellular components. This is more critical and sensitive, especially for the very important part of the brain, which is an active immune site and consists of resident and infiltrating immune cells that affect brain growth, tissue homeostasis, and neural activities (Korin et al., 2018). The brain as the most complex organ of the body, apart from neurons, astrocytes, oligodendrocytes, and glial cells, has its resident immune cells (Schwartz et al., 2013).
Recent studies indicate that identifying and using phenotypic markers are keys to distinguishing different types of immune cells in the brain. Under pathological conditions, Bennet et al. (2016) reported that Ter119 was a distinct and proper marker for distinguishing brain-resident microglia from monocyte-derived macrophages (MoDMs) but it should be noted that the functional analysis is another necessary section of studies on immune components of the brain, which is not limited to the identification of the population of brain immune cells. New studies have found that the same immune cells may play different roles in different stages of diseases. For example, MoDMs increase pro-inflammatory responses in their acute phase in ischemic stroke, but they change to an anti-inflammatory role in the chronic phase (Fang et al., 2018).
Even though the traditional immunology technique, namely live cell sorting or fluorescence-activated cell sorting (FACS), was used in this field, unfortunately, it had limitations in


eISSN 1303-5150

fulfilling the listed very important information needs. To fulfill the growing needs of this field of medical science, phenotypic and functional studies on immune components of the brain need a more accurate and high-dimensional technique such as CyTOF that can simultaneously detect up to 100 parameters (Wang et al., 2022).
Mass CyTOF cytometry (or cytometry by time-of-flight) technique, which has been successfully applied to discover new immune populations in humans and rodents, can enable very high-resolution characterization of dozens of markers at a single-cell level and artistically present a bird's-eye view of the immune system in the body. Therefore, the present study summarized the use of CyTOF for the vital organ of the brain, and the principles, advantages, and disadvantages of this technique.



## 2- Cytometry by time-of-flight (CyTOF)

### 2-1- History of the CyTOF technique

During the 1960s, the collaborative efforts of scientists active in cytology led to the invention of a method called flow cytometry, which made it possible to detect particles, including cells, and evaluate their characteristics. The high speed and accuracy of this method caused it to get a special place in various fields of medical and biological sciences (Mohammadsadeghi et al., 2013). The result of initial efforts in this field was the creation of a single-agent device that only detected the sizes of cells, but today, powerful flow cytometry devices have emerged with the capability of simultaneous detection of up to 14 different factors from hundreds of thousands of single particles per second (Wilkerson, 2012). Analyzing this large volume of data from each test is a very sensitive and specialized task and they are obtained from graphs and charts and analyzed with the help of specific software.
This technique is usually used to diagnose general pathology. It is also applied in the field of medical diagnosis, especially distinguishing and classifying normal blood cells from abnormal cells and diagnosis of various blood malignancies, the examination of the response to treatment in malignancies and AIDS, and measurement of specific factors in diagnosis and prognosis of the disease. It is applicable in the research on rates of cell death and apoptosis/necrosis, the rate of DNA genetic material, the viability of cultured cells and bacteria, the cell cycle process, the determination of the karyotype, etc., It has been emphasized in studies by Jennings, Foon (1997), Landay et al. (1997), Tiersch et al. (1989), Dolezel (1997), and Díaz et al. (2010). The use of high dimensional (HD) flow cytometry to detect more than 40 parameters simultaneously has emerged as a technique for large-scale immune profiling and biomarker discovery (Gadalla et al., 2019).
Cytometry by time-of-flight (CyTOF), which is also known as mass cytometry, is a new combination of flow cytometry and mass spectrometry that excels in multiparametric single-cell analysis. CyTOF allows simultaneous detection of up to 50 parameters per cell through the use of rare metal-labeled antibodies, inductively coupled plasma (ICP) ionization, and time-of-flight detector, according to recent technological and computer advances. (Zhang et al., 2020)
Bendall et al. (2011) used this technique for the first time in the field of immunology and analyzed 34 parameters by single-cell suspensions from human bone marrow to show differential immune cells and drug responses in the human hematopoietic disease. Since then, this technology has also been used in immunological studies. Over time and with the advancement of technology, the number of parameters has increased significantly in the diagnosis panel. This new approach has no fluorophore overlap problems that occur in using the traditional FACS method. (Bandura et al., 2009; Razumienko et al., 2008)

### 2-2- Principles of cytometry by time-of-flight (CyTOF)

The cytometry method aims to individually measure the physical and chemical characteristics of particles. Even though synthetic particles and components isolated from the cell and also particles smaller than the cell can be analyzed with this method, animal, plant, and microbial



cells are often analyzed with this method. After being injected into the device, the cells in the sample are placed in a stream of fluid and pass one by one in front of the laser light beam (Macey, 2007). After colliding with each cell, the light is refracted at different angles. The light rays, which pass along the edges of the cell, have the lowest angle of refraction and are scattered almost forward. This light scattering is called forward scatter (FSC) or forward angle light scatters (FALS) and can be a measure of particle or cell size. The light rays, which pass through the particle or cell, are refracted at larger angles and scattered around due to collision with its internal content. These light scatterings, which are called Side Scatter (SSC) or Right Angle Light Scatter (RALS), are measured at an angle of 90º to the laser light beam and show the degree of intracellular structural complexity, such as its granularity (Figure 1).



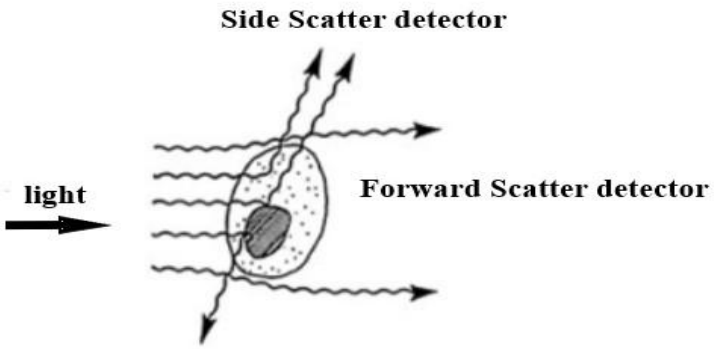


**Figure 1-** Refraction of laser light in the collision with the cell and its scattering in forward and side angles

In this method, the markers or surface and cytoplasmic antigens of the cells are important in addition to the size and structure of the cells. These antigens can be labeled with an antibody attached to fluorescent dye or by using free fluorochrome. The electrons in the molecular structure of the fluorescent dye are excited when they collide with the laser light and they emit a specific range of visible light when they return to the base state. Therefore, the laser light radiation to labeled cells causes the emission of fluorescence. Accordingly, higher intensities of fluorescence emission indicate more presence of antigen in the cell. Finally, the scattered light in FSC and SSC, and also the emission of fluorescence are separated by filters, collected by detectors, and converted into electric current (voltage) with the help of a converter. The electric current is converted into digital data using the processor system that is displayed by the computer as a graph (Shapiro, Telford, 2018). Therefore, as each cell or particle passes through the laser beam, the laser light is deflected and the fluorescent light emitted by the cells is collected, converted into analog signals, and separated by optical filters and mirrors according to different wavelengths. These signals are digitized and transferred to the screen as a histogram. A histogram is a visual representation of the frequency distribution of measured parameters (Dunphy, 2004).

Cells must be alive or resident during the measurement, and each cell must be suspended in the liquid. The suspension with the cells must pass through the laser beam in continuous flow. Each cell deflects some of the laser light and emits fluorescent light because it is excited by the laser, i.e. loaded with extra energy (Laane et al., 2005).

Therefore, cytometry measures several parameters simultaneously for each cell. These parameters include 1- the forward scattering intensity at a low angle and approximately proportional to the cell diameter, 2- the orthogonal scattering density (90°) approximately proportional to the number of granule structures in the cell, and 3- the fluorescent intensity at many wavelengths.



eISSN 1303-5150

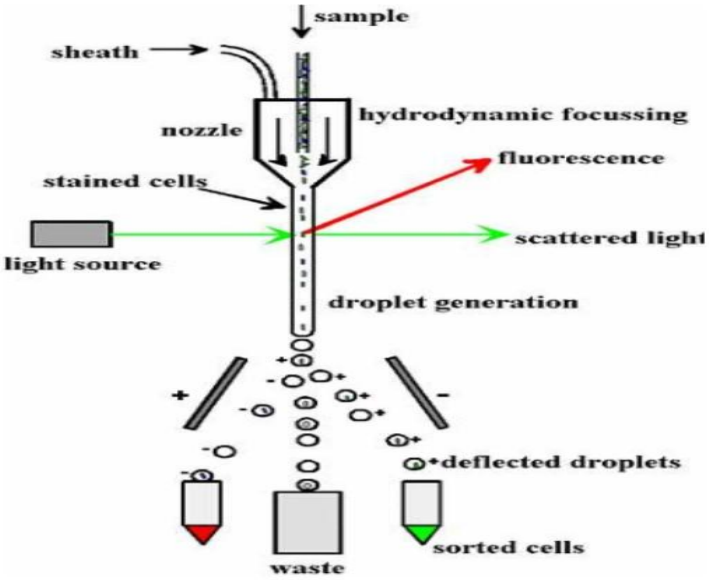


**Figure 2-** Principles of the cytometry technique

Therefore, the most powerful and unique advantage of cytometry is that it can physically distinguish cells from each other. Therefore, pure samples of specific cells can be obtained for further analysis (Daniel, 2004). Such research has been used in stem cell and gene therapy studies since 1990 (Brown, Wittwer; 2000).

### 2-3- The main stages of cytometry analysis

Cytometry technique is a very fast method that provides accurate reports of results by analyzing thousands of cells in a very short time. In a heterogeneous sample population, multi-parameter quantitative properties of cells and their components can be analyzed in a short time (Table 1) (Ibrahim, Engh, 2007).

**Table 1-** The main stages of the cytometry technique

| Implementation stages | Necessary measures |
|---|---|
| Before analysis | - Experimental model design<br>- Sampling with anticoagulants<br>- Providing other samples if necessary<br>- Examining the transportation and storage conditions<br>- Determining the proportionality of samples (e.g. coagulation status and hemolysis)<br>- Identifying the livability of samples (percentage of identifying the dead cell) |
| During analysis | Calibration of Laser in the device; light balance; controlling the light filters; the electronic control; detector, and controlling the voltage; calibration of fluorescent dyes in flow cytometer; the order of control group materials; control granules; biological materials; selecting antibodies; specificity; desire for combination; a panel of materials' antibody in the empirical model; identifying the compounds; analysis of one or more parameters; lid arrangement process; separation and collection of target cells (cell sorting); removing the dead cells |
| After analysis | Interpretation of data<br>Reporting<br>Clinical use<br>Clinical result |

### 4-2- Cytometry technique analysis methods

In flow cytometry, quantitative and qualitative analyses of the cell or its components can be performed in four ways (Brown, Wittwer, 2000; Dunphy, 2004; Herzenberg et al., 2002; Rahman, 2006).
The methods are as follows:
1- Analysis according to the size and structure of the cell granule.
2- Analysis using a monoclonal antibody labeled with a fluorescent dye.
3- Analysis using antibodies labeled with multi-color fluorescent dyes (2-17 dyes).
4- Analysis of antigens and structures in the cell with monoclonal antibodies labeled with the fluorescent dye, which is performed as a result of increasing the cell membrane permeability (Maecker et al., 2005; Wood, 2006).



**5-2- Advantages and disadvantages of the cytometry technique**
This method has great advantages as follows (Langsrud, Sundheim, 2000):
**Speed**: This technique is very fast with the potential of testing thousands of cells per second.
**Sensitivity**: The fluorescence of cells is not bright enough to be measured with a manual epifluorescence microscope but these cells can be detected and measured by flow cytometry.
**Precision**: Stability is less than 1% in coefficient of variation (mean/standard deviation) for measuring light scattering and fluorescence of uniform microspheres.
The most powerful and unique advantage of flow cytometry is that it can physically separate cells from each other based on any optical characteristic or combination of them. Therefore, pure samples of specific cells can be obtained for further analysis. Even though flow cytometry devices have high setup costs, they have low operating and maintenance costs [36].
Furthermore, CyTOF significantly increases the detection capacity. Since this procedure requires barcoding of the samples before the staining step, it can perform the same staining, rinsing, and permeability steps for up to 20 samples as a multiplex composite sample (Mei et al., 2015).
This technique can also provide reliable quantification, as Gadalla et al. (2019) also reported, even with a small number of input cells. During their protocol, it is possible to provide a reliable quantification using CyTOF for a small number of human input cells. Their studies also indicate that this technique can successfully summarize FACS data on PBMCs and tumor tissues and enable reliable staining for more than 35 parameters for high-dimensional analyses in clinical cancer studies. Another significant advantage of this technique is the spectral overlap and background noise that significantly decreases using this technique. Furthermore, the detection overlap between heavy metal isotopes is usually limited to less than 2% in this technique (Leipold et al., 2015) the spectral overlap is from 5% to 100% in a method such as conventional FACS. The traditional FACS also uses fluorochromes as labels, while metal labels all have similar chemical properties in the CyTOF technique because they belong to the lanthanide family and thus increase general sensitivities (Tricot et al., 2015).
On the contrast to these advantages, this technique has disadvantages which are as follows respectively (Dalva, Gülbaş, 2005):
Limited resolution: Flow cytometers can usually measure peak or integrated signals but they cannot provide advanced structural details. Therefore, phytoplankton or their species can be rarely detected while cells are classified based on their optical properties. Other methods such as epifluorescence microscopy and image analysis provide a much greater resolution of heterogeneity in a plankton sample.
Small sample size: Many flow cytometers analyze very small volumes (<0.5 $mm^3$), while the cells are at least about 103/ml. Therefore, they cannot be accurately analyzed without pre-focusing or modifying the instrument. Even though flow cytometers can make very accurate measurements, such measurements will depend on the proper calibration of the sample.



Mass cytometry differs from fluorescence cytometry in various aspects, including dimensions, sensitivity, and cell throughput which are summarized in Table (2) (Zhang et al., 2020).

**Table 2-** Comparisons between flow cytometry and CyTOF properties



| | | **Flow cytometry** | **CyTOF** | |
|---|---|---|---|---|
| | Labeling | Fluorochrome | Heavy metal | |
| | Detector | Fluorescence detector | Mass spectrometry | |
| | Multiplex | Up to 30 | Up to 45 | |
| | Sensitivity | High | Low | |
| | Sample efficiency | >95% | <50% | |
| | Accessibility | Easy | Moderate | |
| | Cell throughput | 10 000 cells/s | 500 cells/s | |
| | Cost | Moderate | High | |
| | Sorting | Yes | No | |
| | Cell recovery | Yes | No | |

**6-2- Phenotype characteristics of the cytometry technique**

According to the above-mentioned cases, CyTOF can multiplex up to 45 cell markers with a limited need for spectral overlap compensation, opening a post-fluorescence cytometry era for deep phenotyping of cells in complex systems. Genetically similar or even identical cells, which play specific roles in disease pathogenesis, can be distinguished by CyTOF. Given the ability of quantitative investigation, CyTOF can detect different cell types, including rare cells throughout the immune chain (Unen et al., 2017).

Current innovations in functional measurements of this technique can be classified into 12 categories: (a) Phenotypic characteristics, (b) Determination of intracellular cytokines, (C) Characterization of intracellular signaling status, (d) Measurement of cell volume and size, (e) Cell viability discrimination, (f) Cell cycle detection, (g) Proliferation tracking, (h) Receptor occupancy assay, (i) Tetramer-based antigen-specific T cell screening, (j) Chromatin modification profiling, (k) Detection of RNA and protein-coding, and (l) Mass cytometry imaging (Zhang et al., 2020).



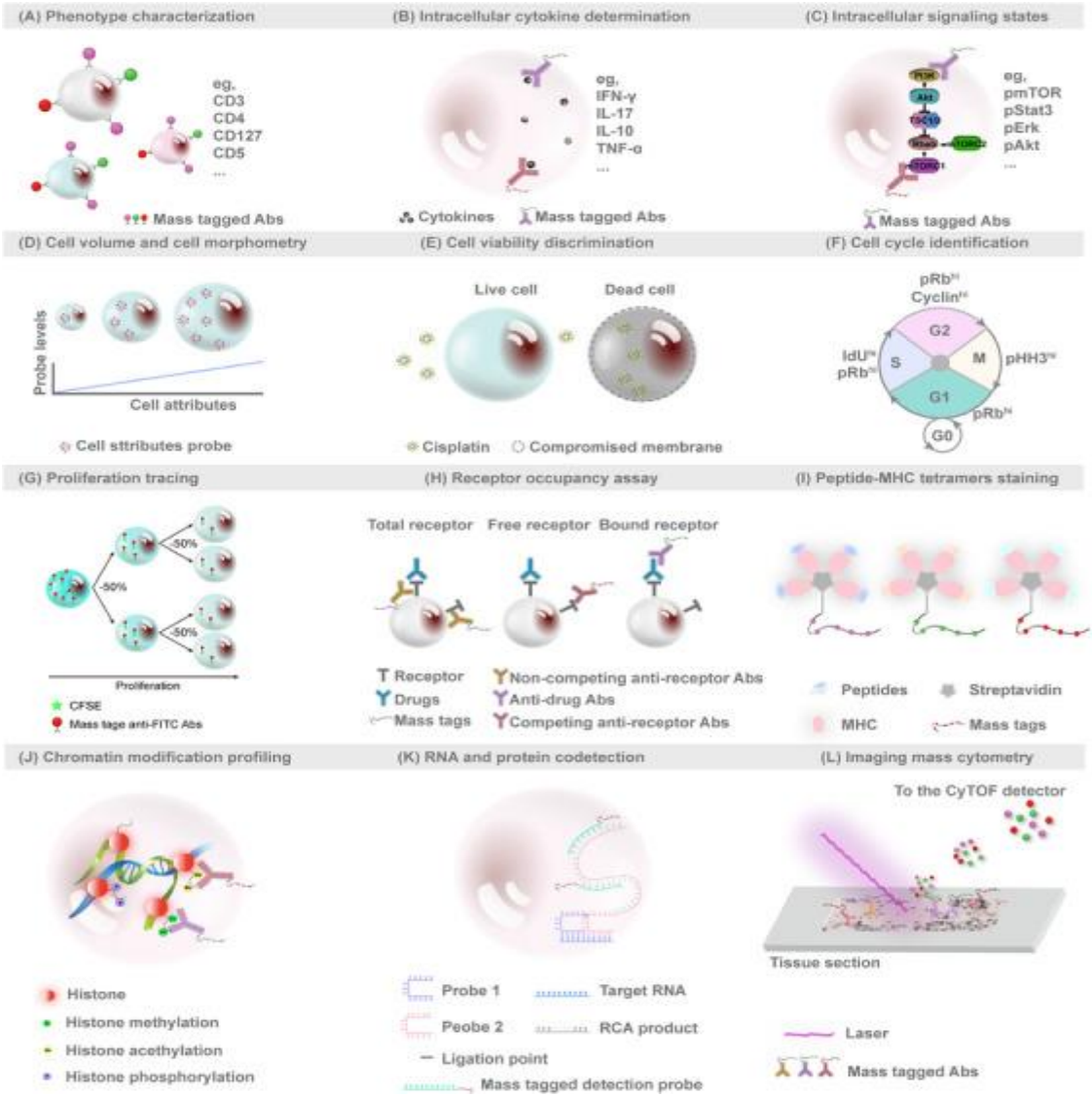


**Figure 3-** Schematic images of functional assays using the CyTOF technique

## 3- Application of cytometry technique in brain studies

There are partially scarce major advances in brain injury research, including apoptosis (Wang et al., 2018), inflammation (Suzuki, 2019), and oxidative stress (Xu et al., 2019) but immunology has received rapid attention (Tong et al., 2017). Despite few available studies, the steps taken are a great success in human science and technology and provide a deep understanding of the immune response after brain injuries. However, few neuroscientists have used these techniques to explore the immune system's effect on brain repair, and we should stay to see how researchers will use these powerful tools to systematically deal with this issue.

Traditional flow cytometry has a limited capacity to detect multiple fluorescence parameters owing to overlapping fluorochrome spectra, but the CyTOF technique, with its high advantages, can assess the immune space of the brain and show the perspective of immune cells both in a simple brain (i.e. healthy conditions) and in pathological conditions (e.g. neuroinflammation and neurodegenerative diseases) (Wang et al., 2022). Figure (4) shows the general procedure of the CyTOF technique in which the necessary samples are collected using the human samples or animal models.



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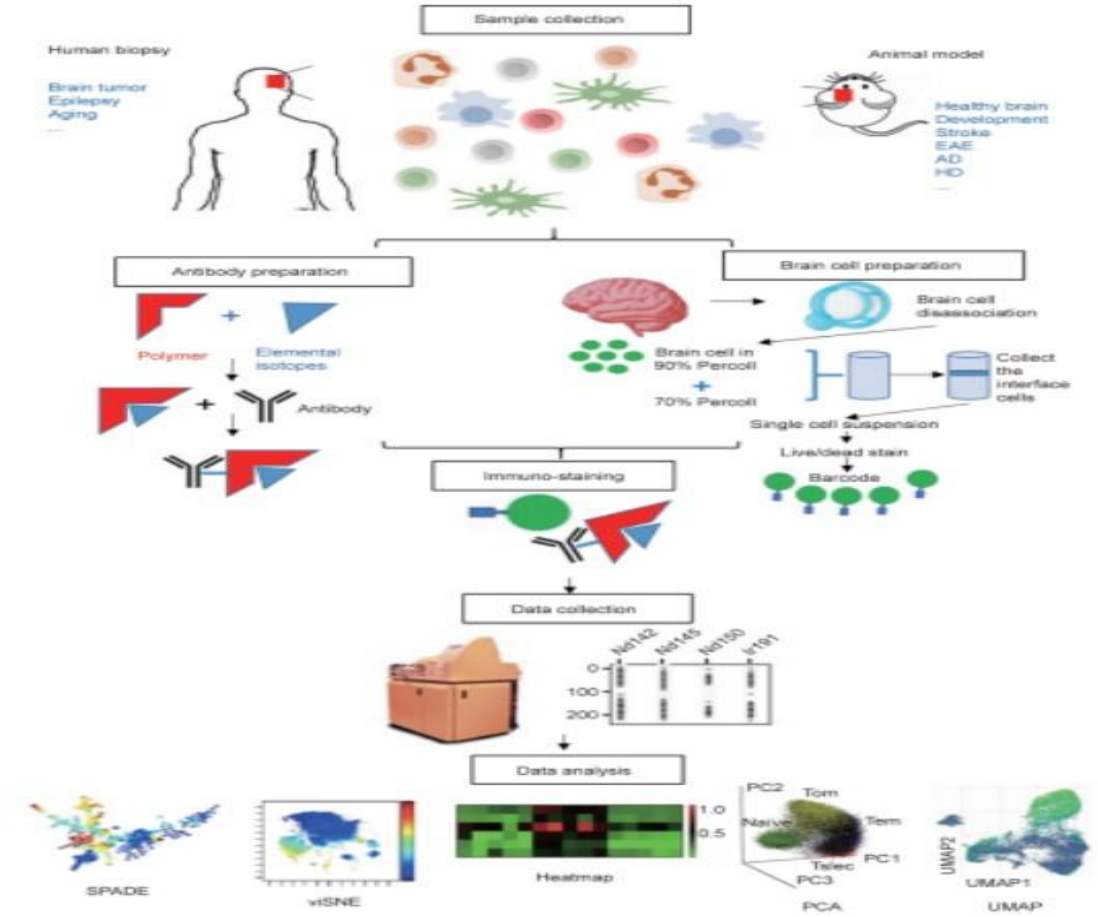


**Figure** 4- The general procedure of the CyTOF technique according to human or animal samples

According to Figure 4, a conjugated antibody with a unique heavy metal isotope is used after sampling to prepare the antibody. These single cells are first stained as alive or dead and then marked with a barcode to prepare a single brain cell suspension.
Single cells are stained with ion-conjugated antibodies. The obtained single-labeled cells are analyzed using a CyTOF device. Data analysis can be performed using Cytobank and assessments can be performed using (but not limited) the Spanning-tree Progression Analysis of Density-normalized Events (SPADE), visualization of high-dimensional single-cell data based on the randomized t distribution, heat maps, principal component analysis (PCA), and uniform manifold approximation and projection (UMAP). In this figure, EAE: encephalomyelitis; AD: Alzheimer's disease; HD: Huntington's disease; Nd: Neodymium; Ir: Iridium, and Nd142, Nd145, Nd150, and Ir191 are also abbreviations for different CyTOF canals. Tom, Tem, and Tslec are abbreviations for different cell types detected by CyTOF.

### 3-1- Cytometry technique in central nervous system studies

According to the results of several studies, the central nervous system (CNS), which consists of the brain, spinal cord, optic nerves, and retina, contains a variety of immune cells with diverse roles in tissue homeostasis, immune defense, and neurological diseases. Mapping leukocytes throughout the brain is challenging, especially in pathology, where phenotypic changes and an influx of blood-derived cells prevent clear differentiation between active leukocyte populations (Mrdjen et al., 2018). Since this central system does not have any classical lymphatic drainage system, the brain is traditionally considered an immune-privileged organ (Ley et al., 2007).
The higher functional and anatomical evidence highlight the vital role of immune activity in the CNS (Louveau et al., 2015). The brain immune compartment contains infiltrating and resident immune cells located in the parenchyma and in non-parenchymal structures such as

perivascular spaces, subdural meninges, and choroid plexus (CP) (Engelhardt et al., 2017). Brain-resident myeloid cells (known as microglia) populate CNS early in embryonic growth and are adapted to the specific needs of this tissue. Resident myeloid cells perform normal myeloid functions such as phagocytic clearance of debris and sensing tissue damage, as well as unique tissue functions such as regulation of neuronal excitability, and synaptic organization (Priller, Prinz, 2014; Heppner et al., 2015).



Infiltrating immune cells, which interact with the brain compartment, undergo a selection process in a way that only certain cell populations are included in the brain's immune space. We can propose models regarding their roles in normal brain functions by determining which immune cells closely surround the nascent brain. Strategies based on gene expression analysis provide unbiased insight into these questions. They provide an in-depth description of the immune populations in the brain and indicate gene expression profiles of myeloid cells in different brain regions under pathological conditions. Mass cytometry (CyTOF) provides a broad and high-dimensional view of the brain's immune space. This technology can detect the surface marker expression of a wide range of immune cell populations at the single-cell level. Like flow cytometry, mass cytometry determines the level of proteins in individual cells. However, unlike flow cytometry, mass cytometry uses antibodies labeled with heavy elements, and thus it is not confounded by the overlapping emission curves of fluorophores and allows the simultaneous measurement of tens of markers in each cell. (Spitzer, Nolan, 2016)

### 3-2- Brain health, neurodegeneration, and CyTOF technique

Immune cells play key roles in maintaining brain functions under healthy conditions. Brynskikh et al. (2007) reported that immune cells directly participate in brain learning. This issue is true in animal studies, especially in rats with immunodeficiency or those with lower immunity. In human studies, Ziv et al. (2019) also indicated that $CD4^+$T cells help maintain neurogenesis and spatial learning in adults. Autoimmune T cells also play vital roles in studies on brain immune cells and functions. In an animal study, Kipnis et al. (2004) indicated that adult rats lacking autoimmune T cells suffered from severe cognitive defects and behavioral abnormalities, but they gradually recovered after T cell regeneration.

The brain and its borders create a very dynamic microenvironment of immune cells; however, the detection of immune cells in the simple brain compartment is very limited (Korin et al., 2017). The assessment of the roles of immune cells in the healthy steady state is technically a bit challenging owing to a very small number of immune cells in healthy brains. This is especially obvious when the traditional FACS approach is used.

RNA sequencing, mass cytometry, and whole-genome transcriptomic and proteomic analysis allow us to have a deeper and broader understanding of the brain's immune system functions and reactions to different pathophysiological conditions. In these techniques, RNA sequencing methods have been helpful in depicting the complexity and diversity of immune cells (Chen et al., 2019).

Multiple nerve inflammations in the brain caused by internal or external stimuli sometimes can cause challenges. The landscape of leukocytes changes dramatically during brain neuroinflammation. However, image-based immunohistochemistry or traditional FACS, which is usually used in these cases, has a low capacity to detect different types of immune cells using a small number of samples. Therefore, CyTOF, as a high-dimensional single-cell detection technique, can comprehensively reveal the leukocyte map and the whole immune landscape during neurodegeneration and neuroinflammation in the brain.

The immune landscape of CNS changes dramatically during neuroinflammation. The resident immune cells are activated and the parenchyma can be infiltrated by inflammatory leukocytes from the environment. Resident phagocytes of CNS must present themselves to encephalitogenic T cells during the experimental autoimmune encephalomyelitis (EAE) by modeling multiple sclerosis (MS) (Schreiner et al., 2009). Several studies by Greter et al.



(2005) and Kivisakk et al. (2009) assigned this role to DCs or BAMs during the onset of EAE. Several studies emphasize that a process called "inflammation" gradually increases in the brain in aging (Deleidi et al., 2015) and CNS leukocytes may also contribute to Alzheimer's-associated inflammation, aging, and neurodegeneration (Baruch et al., 2013; Prokop et al., 2015).

In another study, Ajami et al. (2015) discovered three new $CD11b^+$ brain myeloid cell populations using CyTOF in an EAE animal model (mouse) and named them A, B, and C respectively. All three cell populations expressed conventional markers CD45, CD11b, CD317, and CD39. Their studies indicated that populations B and C still expressed MHCII and CD86 even though they were previously detected as markers of myeloid cell activation in some studies.



**3-3- Neuroinflammation caused by stroke and the CyTOF technique**

Stroke is a brain disorder and one of the most common causes of death worldwide. In modern medicine, blood supply disorder is known as an important determinant of organ dysfunction and is the most important cause of cell and organ death (Simon et al., 2009). Ischemic or occlusive stroke is one of the three common types of stroke, accounting for approximately 80% of stroke cases worldwide (McConnell, Tyska, 2010). Ischemic damage causes important neurological defects, including microvascular dysfunction, damage to the blood-brain barrier (BBB), oxidative stress, and cerebral edema (De Meyer et al., 2016).

Dead neurons release damage-associated molecular patterns (DAMPs), which often cause neuroinflammation in the ischemic hemisphere. This focal inflammation of the brain aggravates secondary brain damage and causes subsequent brain inflammation (Shi et al., 2019).

Nerve damage immediately after stroke stimulates the activity of resident microglia, followed by the infiltration of peripheral circulating leukocytes (Chamorro et al., 2012; Fan et al., 2016). On the contrary, lymphopenia occurs in peripheral immune organs from a few hours to a few weeks (Wang et al., 2020). Benakis et al. (2015) reported that there was a correlation between brain neuroinflammation and immune system suppression in peripheral organs.

Cytometry is a versatile technique for analyzing stroke-induced changes in the immune system. Unlike other cell detection methods such as immunohistochemistry, this technique is fast and highly sensitive and can determine multiple markers in cell suspensions. There are many protocols both for the isolation of immune cells from the brain and for subsequent analysis through cytometry (Amu, 2022).

Full details of these protocols are published to ensure the reproducibility of preclinical and clinical stroke results. "The Minimum Information about a Flow Cytometry Experiment (MIFlowCyt)" guideline was introduced in 2008 (Lee et al., 2008).

Li et al. (2020) utilized the mass cytometry technique (CyTOF) to quantify the number of immune cells in different organs from 1 to 14 days after stroke and analyzed cell surface markers. Their results indicated that CD62L might mediate a specific type of immune cell. In another study, Yao et al. (2021) investigated Ischemic postconditioning (IPostC) as a concept of ischemic stroke treatment using the CyTOF technique. Their results indicated that the reduction of 4E-BP1, Microglial p38α, and MoDM in the ischemic brain plays a very effective role in the protection caused by IPostC. Furthermore, the reduction of p38 of p38 CD4 T cells and Treg also contributed to the protection caused by IPostC in peripheral blood.

Recent studies indicate that the combination of cytometry technique and clinical animal models of brain ischemia provides a broader and deeper image of the multifaceted role of immune cells in the development of stroke (Malone et al., 2019).

Many other researchers such as Wang et al. (2020) Gan et al. (2014), Gu et al. (2012), and Anttila et al. (2007) indicate that various immune cells such as brain resident microglia, infiltrating T cells, monocytes, NK cells, and neutrophils participate in brain inflammation caused by an ischemic stroke.



### 3-4- Brain tumors and the CyTOF technique

Brain tumors include all tumors inside the skull or tumors inside the central canal of the spinal cord. These tumors are usually in the brain, including neurons, glial cells, astrocytes, oligodendrocytes, ependymal cells, myelin-producing cells, Schwann cells, lymphatic tissue, blood vessels, or in cranial nerves, meninge, pituitary and pineal glands. (Fedele et al., 2005)

If the tumor arises from the brain or in tissues close to it, including in the membranes covering the brain (meninges), cranial nerves, pituitary or pineal gland, it is called a primary tumor, and when the tumor spread to the brain from other parts of the body such as the lungs, breast, kidney, or other regions of the body, it is called metastatic (secondary) brain tumors which are different from primary brain tumors. In general, the probability of developing a brain mass due to cancer metastasis in another part of the body is 10 times higher than developing a tumor due to cancer of the brain itself. One out of four cancer patients will usually have metastatic brain cancer (Jayachandran and Hdansekaran, 2013).

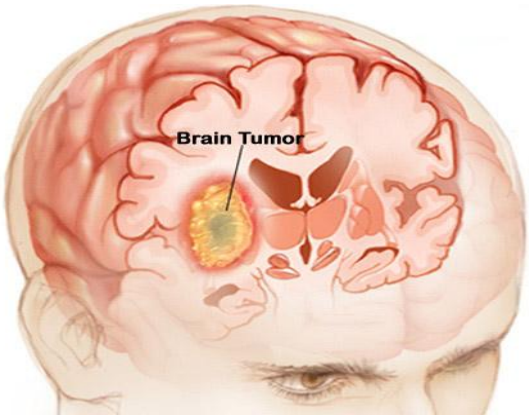


**Figure 5-** Tumor creation in the brain

As the most heterogeneous tissue, the tumor microenvironment (TME) of the brain not only contains the unique resident immune cells of the brain, i.e. microglia, but also various immune cells from the environment. Therefore, the correct definition of brain tumor immunity helps to understand the interactions between tumor and immune cells which can also help to predict the immunotherapy results (Hu et al., 2019).

To detect tumor-specific invasive leukocytes, Becher et al. (2014) used a total of two CyTOF panels and 74 parameters for the comprehensive evaluation of myeloid cells and lineage immune cells using 38 ex vivo surgical gliomas, brain metastases (BrMs), and non-tumor epilepsy samples. The results indicated that glioma and BrMs had distinct TMEs. Tumor-associated macrophages (TAMs) are the predominant immune cells in glioma, while tumor-infiltrating lymphocytes (TILs) are the main immune cells in the brain.

Kawamoto et al. (1979) used low cytometric analysis for DNA content in normal human brain cells and in benign and malignant CNSMs. They found that the loss of heterozygosity was similar to the CNSM stage. Using a small number of samples, they proved the validity of this technique and were the first researchers who proposed the use of cytometry and DNA distribution as data of clinical importance in cancer in general and in the diagnosis of CNSM in particular.

Hoshino et al. (1978) presented similar data on several types of CNSM. The main methodological difference between this method and the above method is the purification of the nuclei obtained from the centrifugation of CNSMs through 40% sucrose and staining with an Acriflavin-Feulgen reagent for DNA analysis. The results were similar because DNA content in benign tumors (meningioma, pituitary adenoma, neuroblastoma, and low-grade astrocytomas) often indicated diploid cell populations with low proliferative index, while



eISSN 1303-5150

most malignant CNSMs, which were often gliomas, had aneuploid populations and/or higher proliferation index.
Another interesting insight was added by Petersen et al. who considered a rare CNSM by flow cytometry and cytogenetic analysis as hypodiploid, containing about 75% of the normal value of DNA of a diploid cell (Petersen et al., 1981). New studies indicate that the use of flow cytometry is useful not only for solid brain tissue but also for the analysis of cerebrospinal fluid in case of infiltration of pathological cells (Helson et al., 1982).

In a study by Peereboom et al. (2019) on glioblastoma (GBM), which is the most common primary malignant brain tumor, they analyzed circulating immune cells through flow cytometry and evaluated the immune profile of treated tumors with mass cytometry (CyTOF). The researchers reported that Myeloid-derived suppressor cells (MDSCs) were present in the circulation of GBM patients and in the tumor tissue and were associated with a low prognosis. Low-dose chemotherapy decreased MDSCs in clinical models, but the use of this strategy to reduce MDSCs in GBM patients was not yet evaluated; hence, it was used in this study. The final results of this study indicated that low-dose metronomic capecitabine in combination with bevacizumab was well tolerated in GBM patients and was associated with a reduction of circulating MDSC levels and an increase in the infiltration of cytotoxic immunity into the tumor microenvironment.
In another study, Friebel et al. (2002) used the CyTOF technique, indicating that TAM was directed by a specific type of tumor. Using the CyTO technique, Fu et al. (2020a) confirmed that glioma-associated microglia/macrophages (GAMs) were the predominant immune cells and contributed to the immunosuppressive properties in primary and recurrent GBM. Unlike the brain, where TAMs accumulated, the number of myeloid-derived cells in brain tumor patients decreased in the environment.

**3-5- Brain synapse and CyTOF technique**

A synapse is a biological structure at the end of all axons through which a nerve cell sends its message to the dendrite, cell body, or axon of another nerve cell, muscle cell, a gland. There are many synapses in the human brain and they can be classified into two types, electrical and chemical. The use of synaptometry by time-of-flight (SynTOF), which is basically the use of the CyTOF technique in brain synapse research, is very useful for understanding the composition of cell-neuron synapses in the nascent brain. Neuron synapses play key roles in signal transmission.
Gajera et al. [76] were the first researchers who adapted the CyTOF technique to analyze human synaptosomes and brain-enriched nerve terminals and called this method “SynTOF”. They stained single cells from collected synapses with a panel of neuronal markers specific for synaptosomes. This marker panel included phenotypic markers specific to brain cell types (e.g. CD11b, CD56, and CD298), myelin basic protein (MBP), and synapses (e.g. CD47, dopamine transporter (DAT)). This method allowed Stanford researchers to compare the human brains without pathological changes or with pure Alzheimer's disease (AD) or Lewy body dementia (LBD)
Using the machine learning approaches to the dataset, this technique has not only confirmed previously-established differences, such as reduced caudate dopamine transporter in LBD and increased hippocampal pathologic tau in AD, but has also provided new insights; for example, increased hippocampal CD47 and reduced DJI protein in AD, and higher ApoE protein in AD with dementia.
Phongpreecha et al. (2021) measured 38 antibody probes in about 17 million single synaptic events from the human brain without pathological changes or with pure AD or Lewy body dementia (LBD), non-human primates (NHPs) and PS/APP mice. Their results indicated that the synaptic molecular integrity was similar in humans and NHPs. Even though Aβ was not detected in human synapses, it was present in single synaptic events of PS/APP mice. Clustering and identification of human synapse patterns indicated the expected differences of


specific diseases such as increased hippocampal pathologic tau in AD and reduced caudate dopamine transporter in LBD. Furthermore, it confirmed the previously unknown findings such as increased hippocampal CD47 and reduced DJ1 in AD, and higher ApoE in AD with dementia.

Gajera et al. (2019) used the SynTOF technique and sought to overcome the challenges of single synapse analysis and analyzed 390,000 individual synaptosomes collected from 13 human brain samples and specified the changes in synaptic diversity in Lewy body disease (LBD), Alzheimer's disease (AD), and normal brain. In this study, they simultaneously analyzed 34 parameters on the collected synaptosomes. The results indicated that this method was associated with expected changes in the molecular composition of striatal dopaminergic synapses in Lewy body disease and Alzheimer's disease. Mass synaptometry enabled highly-parallel molecular profiling of synaptic terminals.



**3-6- Microglia and CyTOF technique**

Microglia, as resident immune cells in the brain, play a major role in brain growth and development, homeostasis, neuroinflammation, neurological diseases, and psychiatric diseases. In diseases, microglia respond to the changed environment and are activated, while their heterogeneity dramatically increases (Wes et al., 2016). In healthy conditions, microglia functionally support the growth of neurons, modify synapses, and destroys dead cells. (Butovsky and Weiner, 2018)

Even though the brain was previously assumed to be an "immune-privileged" organ because peripheral immune cells were unable to cross the blood-brain barrier, today's increasing evidence suggests that glial cells, particularly microglia and astrocytes, form and modulate the complex neuroimmune system of the brain in response to infection and drug intervention (Hidenori, 2019; Antoine et al., 2020). As ongoing research also acknowledges, macrophages, oligodendrocytes, and endothelial cells also play important roles in the neuroimmune process (Voet et al., 2019). The activation of these nerve cells may damage healthy neurons and lead to brain damage by releasing pro/anti-inflammatory cytokines and reactive oxygen species (ROS). It is worth noting that this system is also important for brain metabolism and development by regulating synaptogenesis, neurotransmitter transmission, and nutritional support of neurons (Wynn et al., 2013; Zmora et al., 2017). Therefore, brain immunity may play an essential role in the pathology of several neurological disorders and brain growth.

It is noteworthy that the immune compartment of the brain includes not only resident immune cells- i.e. microglia, but also infiltrating immune cells. In this regard, Shechter et al. (2018), reported that immune cells can enter certain places, such as the brain, which have immunity privilege, through the vascular coating that acts as an immune diversion gate. In another study, Mrdjen et al. (2018) also prove that there are significant values of leukocytes in normal CNS, including brain resident microglia and macrophage-like cells, classical DCs (cDCs), pDCs, B cells, NK cells, and natural killer T (NKT) cells.

In a study by the CyTOF method, Becher et al. (2014) identified microglia, DCs, and monocytes as separate populations in nascent brains that were expanded with the help of cell and protein surface phenotype markers. These data were later confirmed by Mrdjen et al. (2018).

In addition to GBM, the CyTOF technique has also been used for diffuse astrocytoma (DA) and oligodendroglioma (OG). According to Fu et al. (2020b), high-dimensional analysis is urgently needed since the immune microenvironment is important for the development, progression, and prognosis of anaplastic glioma (AG) and this complex environment has not yet been fully identified. Therefore, the researchers analyzed the immune cells of 5 patients with anaplastic astrocytoma, IDH-mutant (AAmut), and 10 patients with anaplastic oligodendrogliomas, IDH-mutant, p/19q (AOD), and their paired peripheral blood mononuclear cells (PBMCs). A panel of 33 biomarkers indicated tumor-induced immune



changes in the AG immune microenvironment. Their study confirms that mononuclear phagocytes and T cells are the most abundant immunocytes in the immune microenvironment of AG. Glioma-associated microglia/macrophages in both AAmut and AOD samples showed highly-immunosuppressive properties.

Based on the highly heterogeneous characteristics of microglia and using single-cell sequencing, Sankowski et al. (2019) and Masuda et al. (2019) studied the spatial and temporal heterogeneity of mouse and human microglia in two separate studies. Their results indicated that different genes are regulated differently during developmental and disease stages in microglia. Their results showed abnormal expression of microglia RNA in human glioma and MS. Sankowski et al. (2019) combined both single-cell sequencing and CyTOF methods, indicating that glioma-associated microglia had disease-associated signatures. They used gene ontology enrichment analysis and indicated the most significant changes that were closely associated with inflammatory responses (e.g. cell-to-cell leukocyte adhesion, response to interferon (IFN)-c, and phagocytic capabilities), and response to oxidative stress.



## 4- Conclusion

The cellular network of the human brain is highly dynamic and complex, but well organized and controlled. Its cellular dynamics pass between separated and integrated states over time in different types of cells and regions. Myeloid cells of CNS include microglia and CNS-associated macrophages (CAMs) as well as infiltrating monocytes all of which are key players in this network.

It is challenging to investigate cellular markers and their phenotypes at the single-cell level in complex heterogeneous tissues, especially in the brain. Peripheral leukocytes migrate to the brain through the BBB during neuroinflammation and thus create a more complex brain microenvironment. The use of the high-dimensional CyTOF technique makes it possible to "see" the whole picture of different immune cells, tumor cells, or synapses at the single-cell level by significantly increasing the number of its staining channels using a smaller number of samples. This technique has created a new insight into the most complex organ of the body, namely the brain, and has created a correct understanding of the diagnosis of brain diseases and the process of their recovery or treatment in recent years.

Applying this technique is especially important for the brain, which is an active immune site and consists of resident and infiltrating immune cells that affect brain growth, tissue homeostasis, and neural activity.

CyTOF can be integrated with other techniques for comprehensive analysis because it allows single-cell analysis of post-degradation changes in proteins that provide new insights into cell signaling dynamics in target cells. Therefore, this technique has been successfully used to discover new immune populations among humans and rodents. Given the very diverse applications of this technique for the analysis of various types of cells in parts of the brain, its use is very useful and usable and helps to save or improve the lives of many people.

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