Chapter 2

2 High-resolution transcriptomic and proteomic profiling of microglia heterogeneity in MS

Anneke Miedema[1], Marion HC Wijering[1], Bart JL Eggen[2], Susanne M Kooistra[2]

Department of Biomedical Sciences of Cells & Systems, Section Molecular Neurobiology, University of Groningen, University Medical Center Groningen, The Netherlands.

[1,2]These authors contributed equally

Keywords: multiple sclerosis$_1$, microglia$_2$, heterogeneity$_3$, spatial$_4$, sequencing$_5$.

Adapted from Miedema et al. Frontiers in Molecular Neuroscience, 2020.

Abstract

Microglia are important for central nervous system (CNS) homeostasis and first to respond to tissue damage and perturbations. Microglia are heterogeneous cells; in case of pathology, microglia adopt a range of phenotypes with altered functions. However, how these different microglia subtypes are implicated in CNS disease is largely unresolved. MS is a chronic demyelinating disease of the CNS, characterized by inflammation and axonal degeneration, ultimately leading to neurological decline. One way microglia are implicated in MS is through stimulation of remyelination. They facilitate efficient remyelination by phagocytosis of myelin debris. In addition, microglia recruit OPCs to demyelinated areas and stimulate remyelination. The development of high-resolution technologies to profile individual cells has greatly contributed to our understanding of microglia heterogeneity and function under normal and pathological conditions. Gene expression profiling technologies have evolved from whole tissue RNA sequencing towards single cell- or nucleus sequencing. Single microglia proteomic profiles are also increasingly generated, offering another layer of high-resolution data. Here, we will review recent studies that have employed these technologies in the context of MS and their respective advantages and disadvantages. Moreover, recent developments that allow for (single) cell profiling while retaining spatial information and tissue context will be discussed.

2.1 Introduction

A range of microglia phenotypes have been reported, possibly with distinct molecular and functional signatures, although these have not yet been fully elucidated. In MS, demyelinated areas can be classified as preactive, active, chronic active, inactive and remyelinated lesions, based on the degree of inflammation, demyelination and axonal degeneration[1]. This lesion heterogeneity is associated with alterations in microglia phenotype and functionality, giving rise to different microglia subtypes. However, the exact role and functions of different microglia subtypes in MS and other CNS diseases is largely unresolved. Also under healthy conditions several factors contribute to microglial heterogeneity, for example the CNS region microglia reside in. Different subtypes of microglia are found in grey matter (GM) versus white matter (WM)[2–4]. Considerable variation in microglia subtypes and density has been described between brain regions as well. In 1990, Lawson et al. observed variation in the density of microglia processes with a more than five-fold difference between brain regions. Furthermore, density variation was even observed within one region[5], illustrating the importance of regional analysis. Why certain brain areas have a higher microglia density than others, is not yet understood. In addition to microglia heterogeneity within an individual, microglia also differ between species and between males and females, which until now mainly has been studied in mice and humans[6–13]. Furthermore, differences in the microglia transcriptome are observed when comparing microglia from various brain regions, and it has been shown that these regions are non-uniformly affected by aging[14]. Therefore, studying microglia heterogeneity requires an appropriate study design. Recently, high-resolution transcriptomic analysis methods, such as single-cell sequencing, contributed to a better understanding of microglia heterogeneity during development, in health and disease[15–20].

Although these studies provided important insights and identified novel microglia subtypes, the lack of spatial information remains a major limitation. In the past few years, technologies for the analysis of gene expression have been developed that add spatial information to gene expression data. Furthermore, single-cell proteomic methods, such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), are evolving. These methods could provide more insight into (regional) microglia heterogeneity under healthy circumstances and in case of CNS disease. Here, we will review recent studies in the context of MS that have implemented such methods and discuss their respective advantages and disadvantages.

2.2 Microglia in MS

2.2.1 White matter microglia lose their homeostatic profile upon neuroinflammation associated with MS lesions

Neuroinflammation in the CNS is associated with a loss of the homeostatic microglia profile, which has been observed in various neuroinflammatory diseases[21]. Beaino et al. have shown that in mice the local neuroinflammatory environment affects microglia activity by downregulating the expression of the homeostatic microglia marker *P2ry12.* Using human microglia in vitro, they identified that a proinflammatory environment decreases *P2RY12* expression. This is supported by data that show a decrease in *P2RY12* expression in normal-appearing white matter (NAWM) and minimal *P2RY12* immunoreactivity in active lesions in post-mortem human MS tissue[22]. The same pattern of expression was seen for the homeostatic microglia marker *TMEM119*[23,24]. Interestingly, *P2RY12* reappeared in mixed active-inactive white matter lesions. This study revealed that mRNA levels of *P2RY12* and *TMEM119* are regulated by interleukin-4 (IL-4) and

IFNγ. In contrast to white matter lesions (WML) and NAWM, levels of *P2RY12* and *TMEM119* did not differ between grey matter lesions (GML) and normal-appearing grey matter (NAGM). This could be explained by the lower number of lymphocytes observed within GMLs compared to WMLs, as lymphocytes secrete inflammatory mediators such as IL-4 and IFNγ and thus indirectly regulate *P2RY12* and *TMEM119* expression[23].

2.2.2 Microglia in MS lesion pathology

Lesions are often classified by the presence/absence of certain proteins to indicate de- or remyelination and/or inflammation. To classify these lesions, IHC can be performed using the inflammation marker HLA-DR and/or CD68 and a myelin marker, such as myelin proteolipid protein 1 (PLP1). Preactive lesions can be recognized by nodules of activated microglia (elevated levels of HLA-DR and CD68) in the absence of demyelination[25]. These clusters of activated microglia express e.g. TNFα and interleukin-10 (IL-10), which both play a role in cell survival, while IL-10 exerts also anti-inflammatory effects and is important for neurogenesis[25–27].

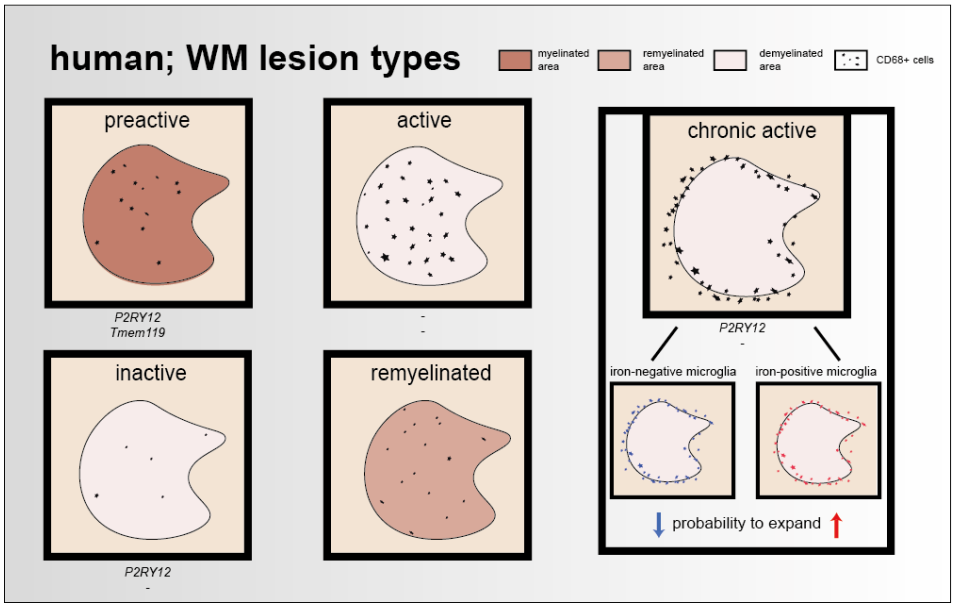

Figure 1: Illustrative overview of different human WM lesion types. Preactive lesions express the homeostatic microglia markers P2RY12 and TMEM119, while expression of these markers is minimal/absent in active lesions and reappears in chronic active lesions and inactive lesions; for remyelinated lesions, the expression of these genes remains unidentified. In each lesion type, CD68+ cells are represented, either within the lesion or at the rim of the lesion. The rim of chronic active lesions can either contain iron-positive microglia/macrophages resulting in a higher probability for lesion expansion or iron-negative microglia/macrophages, which often results in smaller lesions over time.

Within these lesions, microglia have a ramified morphology and express the homeostatic markers P2YR12 and TMEM119, reflecting a (partly) homeostatic state (Figure 1).

Active lesions are characterized by a demyelinated core containing an abundance of foamy myelin-containing microglia within the lesion, facilitating repair. Another hallmark of active lesions is disruption of the blood-brain barrier (BBB), paired with leukocyte infiltration into the CNS[1, 28]. In the healthy brain, oligodendrocytes and myelin are depositories of iron, an essential nutrient for the regulation of myelination and oxidative phosphorylation[29]. However, in active MS lesions, oligodendrocytes are vulnerable to the inflammatory environment and when

damaged, release iron into the extracellular space, leading to the generation of reactive oxygen species (ROS) and uptake of iron by microglia and macrophages. As a consequence, reactive microglia start synthesizing ROS, resulting in local oxidative stress, DNA damage and neurotoxicity[29–31]. These iron-laden microglia have the tendency to stay in this proinflammatory state, impairing clearance of myelin debris, making it harder for oligodendrocytes to migrate towards the lesion site and, as a result, complicates remyelination processes[32-33]. Compared to active lesions, mixed active/inactive (chronic active) lesions contain significantly fewer infiltrated immune cells (Figure 1). Chronic active lesions can be recognized by a rim of HLA-DR positive cells surrounding the demyelinated lesion. These reactive microglia contain phagocytosed iron and other phagocytosed products such as myelin and neuronal debris, which contribute to their amoeboid phenotype[34]. Dal-Bianco et al. visualized these lesions using magnetic resonance imaging (MRI) and showed that lesions with an iron-positive microglia/macrophage rim have a higher probability to expand than lesions with an iron-negative rim which often become smaller, probably due to remyelination. This could indicate that lesions with iron-positive rims impair remyelination[35]. Over time, the infiltrated immune cells disappear, resulting in a chronic silent lesion (inactive lesion). The majority of microglia in inactive lesions are positive for the homeostatic marker P2RY12 but these cells also still express pro-inflammatory factors (Figure 1)[22,24].

2.2.3 Remyelination in MS

Cells from the oligodendrocyte lineage are important for remyelination within lesions. Remyelinated lesions show microglia polarization from a pro-inflammatory state towards an anti-inflammatory phenotype, necessary for the initiation of remyelination[36]. First of all, a pro-inflammatory state is initiated to clear myelin debris and to stimulate oligodendrogenesis in freshly-formed lesions. When lesions expand, phagocytes turn into a more anti-inflammatory state and produce factors (IL-4, IL13 and IL-10) required for OPC differentiation[36–38]. This results in the demyelinated and remyelinated regions that are often detected in/around active lesions. In remyelinated lesions, so-called shadow-plaques, iron accumulation is absent, suggesting that microglia present in these lesions have the capacity for myelin clearance, enabling the maturation of OPCs, which results in the ability to remyelinate axons[35, 39]. Lampron et al. confirmed this by impeding myelin clearance through deletion of *CX3CR1*, a receptor involved in microglia-neuron crosstalk, which resulted in inefficient remyelination of axons[39]. Overall, microglia are very dynamic and the environmental heterogeneity between and within MS lesions stimulates microglia to respond differently. Moreover, iron-positive microglia/macrophages that form a rim around the lesion could impair the remyelination process, resulting in slowly-expanding lesions rather than shrinkage of the lesion.

2.3 Heterogeneity of microglia in the CNS

For a long time, research focused on WM pathology as it was considered the main pathological characteristic of MS. However, demyelination and lesion formation occurs in both the WM and GM of the CNS[40]. In the last decades, studies expanded to include the GM due to advances in visualization technologies and IHC. Similar to WM lesions, cortical GM lesions can be subdivided into different lesion types; leukocortical (lesions that extend in subcortical WM), intracortical (lesions that do not extend to subcortical WM or the surface of the brain) and subpial lesions (lesions that extend to the surface of the brain). It has been shown that WM lesion load is related to cortical lesions; leukocortical and intracortical lesions correlated with incidence of chronic

active lesions, reactive site load and the proportion of remyelinated lesions. However, no correlation was detected between WM lesion load and subpial lesions, indicating that subpial lesion formation is initiated by different underlying processes than in leukocortical and intracortical lesions[41]. Interestingly, subpial lesion formation is often located close to sites of meningeal inflammation, which might contribute to GM lesion pathology[42]. Moreover, differences in microglial phenotype, as well as transcriptomic differences have been observed between species, and males and females. Here, we focus on WM/GM microglial heterogeneity and microglial differences between species and sex (summarized in figure 2).

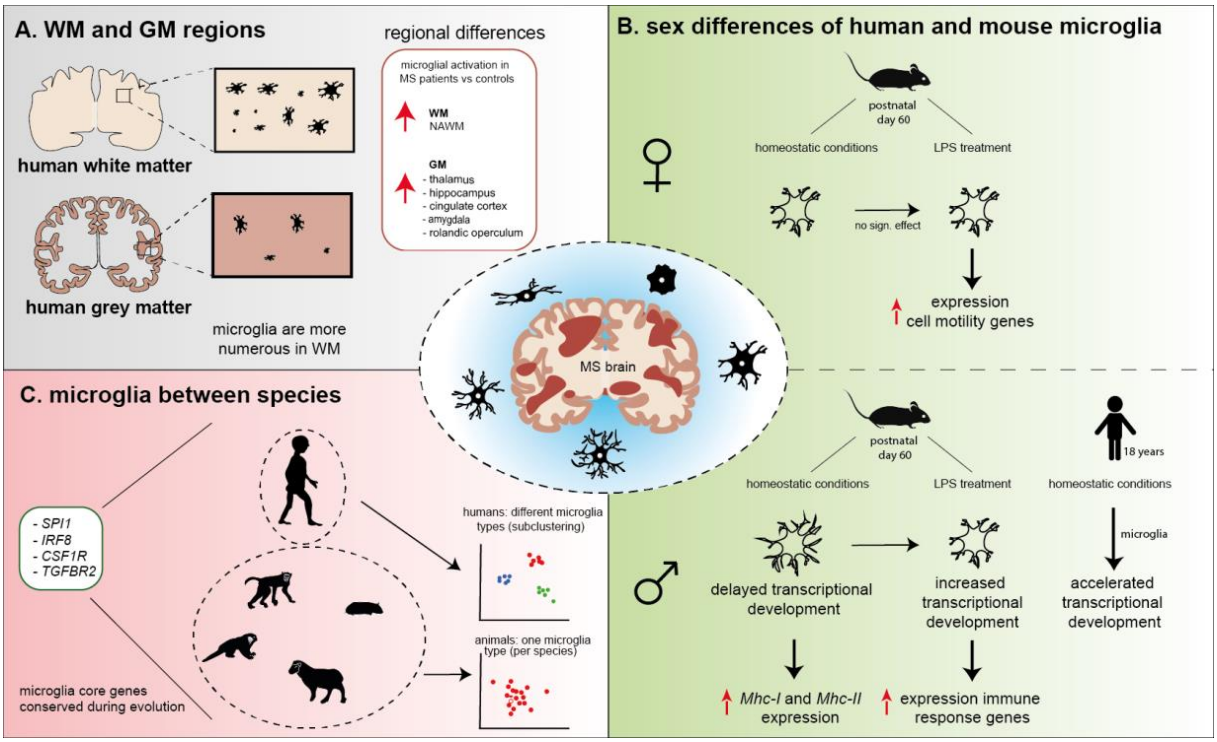

Figure 2: Schematic overview of microglial heterogeneity (A) in WM versus GM regions, (B) between human and mouse male and female microglia, and (C) between species. (A) Microglia in WM are more numerous compared to GM. Microglial activation is observed in NAWM, while in GM, microglial activation is conserved to specific areas. (B) Under homeostatic conditions, mouse male microglia have a higher process volume, process area, number of branches, and number of intersections compared to female microglia. These male microglia appeared to be delayed in the transcriptional development compared to female microglia and show increased expression of *Mhc-I* and *Mhc-II*. LPS treatment decreased male microglial total process volume and area, whereas in female individual, no significant effect of LPS on microglia morphology was observed. In response to LPS, male microglia increased their transcriptional development to a level that was comparable to the transcriptional development in female microglia observed prior to the LPS challenge and increased expression of immune response genes, while female microglia increased the expression of cell motility genes. In opposite, homeostatic human male microglia have an accelerated transcriptional development in comparison to female microglia. (C) *Spi1*, *Irf8*, *Csf1r*, and *Tgfbr2* are core microglial genes that are conserved during evolution, since these genes were strongly expressed in human, macaque, marmoset, sheep, mouse, and hamster. Human microglia subcluster into different microglial types, while mouse, macaque, marmoset, hamster, and sheep microglia grouped mainly into one microglia type, based on their gene expression profiles.

2.3.1 Microglia are heterogeneous across brain regions and differ in WM compared to GM tissue

Microglia are present in both the WM and GM, however these microglia are phenotypically different. Previous studies reported that microglia differ in density, morphology and transcriptomic signature throughout the CNS, which could contribute to the difference in lesion pathology observed in WM versus GM[4,5,23,43–46]. Furthermore, it has been observed that microglia

show a region-specific gene expression profile; GM microglia were enriched for genes involved in type-I IFN responses, while WM microglia expressed high levels of genes involved in the Nf-κb pathway[4]. Transcriptional differences were also observed in NAWM compared to NAGM; NAWM microglia increased the co-expression of genes involved in glycolysis and metal ion homeostasis (*ABCB6, CCR2, LPAR6, SDC1* and *SLC25A37*), whereas in NAGM microglia increased the co-expression of genes associated with lysosomal pathway, lipid catabolism and foam cell differentiation (*ASAH1, CTSD, SCARB2, ABHD2* and *LPL*).

Generally, microglia are more numerous in human WM than in GM (Figure 2)[45], in contrast to mice, where a higher number of microglia is present in the GM[5,47]. However, microglia density is not homogeneous between specific WM and GM regions. In mice the highest microglial density was detected in the frontal cortex, olfactory bulb, basal ganglia and substantia nigra (regions low in myelin), while intermediate microglial density was detected in the cerebral cortex, thalamus and hypothalamus and microglial density was the lowest in the brainstem, cerebellum and fiber tracts (myelin rich regions)[5]. To determine whether microglia from WM or GM respond differently to the same lesion type, Cătălin et al. induced local tissue damage in both the WM (spine) and GM (cortex) by using high-power laser pulses. The morphological response of WM and GM microglia did not differ upon this damaging cue. Although no difference in microglia morphology was identified, the speed at which microglia processes were sent towards the lesion site (μm/min) was faster in WM compared to GM[48]. Furthermore, Verdonk et al., used a CX3CR1GFP/+ model (mice in which the CX3CR1 promoter drives expression of a GFP reporter gene) and treated one group with lipopolysaccharide (LPS). 24 hours after LPS treatment, Verdonk et al. observed morphological differences in brain-derived microglia between the two groups; the cell body and cytoplasm area were significantly larger in microglia from the LPS group compared to controls. Interestingly, in both the LPS and control group the cytoplasm area was significantly larger in the cerebellum compared to frontal cortex, hippocampus and striatum. No significant differences were observed in cytoplasm area between these last three brain regions[49]. Summarized, microglia are not uniformly distributed throughout WM and GM brain regions. Microglia change their phenotype in response to immune modulatory stimuli, however, differences are observed between brain regions.

2.3.2 Regional differences in a cuprizone mouse model

Cuprizone mouse models are frequently used to study the effect of demyelination in WM and to a lesser extent, also in GM. Extensive demyelination occurs within the corpus callosum (CC), however also GM areas such as the cortex are affected by demyelination[50]. A previous study from Gudi et al. showed that microglia start to accumulate in the CC 3 weeks after the start of the cuprizone diet and reach their peak at 4.5 weeks of treatment. In addition, a significant increase in the number of activated microglia was identified within the cortex, however the increase was much lower compared to microglia accumulation within the CC[51]. Interestingly, heterogeneity in microglial distribution throughout the cortex was observed; at 3 weeks of cuprizone treatment, microglia mostly resided in cortical layer V, while at 4-4.5 weeks of treatment microglia were equally distributed through both cortical layers V and VI. At week 5-5.5, the highest microglia number was observed in cortical layer VI, while at all these timepoints demyelination was present in all cortical layers. Oligodendrocytes were also higher in number in these two layers of the cortex 4.5 weeks after the initiation of the cuprizone diet[51]. Cortical layers V and VI are most densely myelinated and are in proximity to the CC, which is one of the regions most affected by demyelination induced by cuprizone, possibly explaining the increase in microglia in these GM regions[51, 52]. Another observation was increased microglial proliferation in both the CC and

cortex during the demyelination phase. However, the number of proliferating cells decreased again during remyelination in both the WM and GM[51, 53]. Most cuprizone studies include young mice that receive a cuprizone diet for 5 weeks to investigate remyelination. In aged mice (6-months of age), 5 week cuprizone treatment did not result in complete demyelination, however, complete demyelination was achieved following 6.5 weeks of cuprizone treatment (0.4% cuprizone) with high accumulation of microglia within the CC. Interestingly, these mice showed incomplete remyelination, with increased numbers of microglia and prolonged microglial activation in the CC, even 3.5 weeks after withdrawal of cuprizone, which could suggest that aged microglia are less capable of phagocytosing debris, resulting in the delayed and incomplete remyelination seen in aged cuprizone mice[54]. Thus when studying MS pathology, the use of an aged cuprizone mouse model should be considered, as this offers more reliable insights in de- and remyelination processes than the young cuprizone mouse model that is most commonly used.

2.3.3 Microglia differences between species

To study MS, cuprizone and EAE mouse models are commonly applied[55, 56]. However, animal models do not fully approximate or recapitulate human disease and pathology. First of all, when comparing mice to humans we should take into account that the environment laboratory mice reside in is clean and controlled, while for humans the environment changes frequently and contains microbes which could cause infections. Furthermore, genetic variation and environmental factors contribute to donor variation, while mice are generally inbred and most factors are held constant throughout an experiment. Here, we will discuss the differences in microglia phenotype and transcriptomic signature between species, with a focus on mice and humans.

Microglia are present within the brain in different morphologies, depending on their environment. Microglia from different species have a similar morphology but there are certain differences. Longer process lengths were observed in human microglia compared to mice[10]. When comparing cingulate cortex microglia from healthy mice and humans[57], a mix of ramified, primed, reactive and amoeboid microglia was reported in humans, while in mice only ramified microglia were observed. Furthermore, a high variation in cell body shape was observed in mice, while in humans the cell body of ramified microglia had a relatively round shape[11].

Gosselin and colleagues compared mouse and human microglial transcriptomic data using bulk RNA sequencing. Mice and humans showed an extensive overlap in microglial gene expression patterns (Pearson's r=0.806), although, genes involved in the regulation of the complement system and brain structure development were expressed at a higher level by human microglia[12]. Recently, a microglial gene expression profiling study of eight species across evolution; human, macaque, marmoset, sheep, mouse, hamster, chicken and zebrafish, using a combined single-cell and bulk sequencing approach was reported[58]. Since chicken and zebrafish microglia showed evolutionary distance to the other six species, these two species were removed from further analysis allowing the characterization of subtle microglial changes between the mammalian species. Hierarchical clustering showed that the macaque transcriptomic signature was most similar to the human transcriptomic signature. Interestingly, all six species showed strong expression of *SPI1, IRF8, CSF1R* and *TGFBR2*; genes with a critical role in microglial development, suggesting that these genes are core microglial genes that are conserved during

evolution (Figure 2). In total, 163 genes were detected that were both conserved and specific for microglia. Moreover, it was observed that human microglia sub cluster into different microglia types, based on their gene expression profiles, while mouse, macaque, marmoset, hamster and sheep microglia grouped mainly into one type (Figure 2). One such human microglial subtype was identified by high expression of inflammatory genes, which could be a result of the effect of aging[10].

Our group previously identified that natural aging affects microglia differently between mice and human. Just a small number of gene expression changes induced by aging are shared between mice and human and quite a few genes show opposite expression patterns[13]. Of note, though microglia age differently under healthy conditions, upon damage or disease, mouse and human microglia show very similar responses[59]. The microglia population is locally maintained by self-renewal. In adult rodents, microglia proliferate every ~95 days. However, the microglia turn-over rate is unequal throughout the CNS and appeared to be higher in the dentate gyrus, where microglial proliferation declines faster with age compared to other brain regions. This could lead to an earlier-reached state of microglial senescence in this brain region, as a result of the Hayflick phenomenon, which describes that cells have a limited capacity to divide due to telomere shortening; most cells divide around 40-60 times[60, 61]. This could explain the significant decrease in microglial proliferation rate that was observed in the dentate gyrus during aging. In humans, microglial proliferation rate has been estimated to be 2.9x higher compared to rodents[61]. Furthermore, humans have a much longer lifespan than mice. When studying age-related brain diseases, it is important to take these differences into account, as they might influence the translational value from studies in mice to the human situation.

2.3.4 Microglial sex differences

Females are at a two to three times higher risk to develop MS and suffer from a higher number of relapses than men[62, 63]. It was observed that MS characteristics are, among others, related to sex differences [41,64]. In relapsing-remitting MS (RRMS), males progress significantly faster than females[64]. In males, a higher incidence of chronic active lesions was observed compared to females. In addition, cortical lesions occurred more frequently in males than in females. However, a direct relationship was observed between the presence of cortical lesions and disease severity in females, while, interestingly, no such relation was observed in males. Another observation was that the presence of chronic active lesions positively correlated with disease severity and lesion load[41], which could explain why in RRMS patients a faster progression is observed in males compared to females.

So far, most research has focused on sex differences in T cell immune responses in MS[65]. However, sex-specific differences have also been described for microglia. During embryonic and early postnatal development, mouse male and female microglia develop in a similar way based on gene transcription analysis. However, a significant difference in the microglial transcriptome of male and female microglia was observed in early adulthood (postnatal day 60). Female microglia appeared to be more mature than male microglia[6]. When early adult mice were injected with LPS 2 hours prior to isolation, changes in morphology, as well as at the transcriptional level were observed in microglia. LPS treatment decreased male microglial total process volume and area, which was inversely correlated with microglial developmental maturity, whereas in females no significant effect of LPS on microglial total process volume and area was observed (Figure 2)[6]. Also sex-specific gene expression responses were identified; male

microglia adapted their transcriptome to a more mature state, which was comparable to the level of female microglial maturity observed prior to the LPS challenge. LPS treatment increased the expression of immune response genes in male microglia, while in female microglia, cell motility genes were highly expressed (Figure 2). Female mice go through cyclic hormonal changes, however, different phases of the estrous cycle did not result in altered gene expression in microglia[6].

Interestingly, in humans, male microglia seem to be more mature than female microglia (Figure 2)[6]. Hanamsagar and colleagues hypothesized that this mouse-human difference could be a result of the relatively sterile environment mice reside in. They postulate that males could be more susceptible for infectious agents during development than females, pushing the transcriptome towards a more mature state[6].

Morphological differences were found when comparing microglia in early adult male and female mice[6–9]. Differences in microglial process volume, process area, number of branches and number of intersections were observed, which were all increased in males compared to females (Figure 2)[6]. Guneykaya et al. also describe an increase in microglial density in the cortex, hippocampus and amygdala in 13-weeks old male mice. Furthermore, an increase in microglial soma size was seen in these three brain areas in adult male mice. These findings suggest that male microglia are in a more primed state and are prepared to directly react to immune modulatory stimuli. This is supported by a higher expression of proteins involved in toll-like receptor (TLR)-pathways found in male microglia, as determined by mass spectrometry-based proteomics[7]. In addition, *NF-κB* transcription activity was 2.4-fold higher in adult male microglia, also indicating a higher responsiveness to immunological stimuli[8]. Thereby, adult male microglia have a higher antigen-presenting potential than adult female microglia. Using flow cytometry analysis, male microglia showed higher expression of MHCI in the hippocampus and amygdala, whereas MHCII expression was increased in the cortex, compared to the expression of MHCI and MHCII in female microglia[7].

The different transcriptomic and functional properties and the immature state of human female microglia could be one of the factors contributing to the higher risk of MS in females. In the last years, research groups have studied sex-specific features of microglia and their response to immune modulatory stimuli. However, less is known about the effect of sex differences in microglia in disease. Therefore, more research is needed to determine if microglial sex differences contribute to the development and progression of neurodegenerative diseases, such as MS.

2.4 Analysis of neuroinflammation in the brain

In addition to the differences observed in microglial morphology in WM and GM, microglia change their phenotype during neuroinflammation. One method to analyze neuroinflammation is TSPO (translocator protein) PET imaging. TSPO is present at the mitochondrial membrane in healthy brains, with increased expression in reactive astrocytes and pro-inflammatory microglia/macrophages[66–68]. Vowinckel et al. showed high concentrations of TSPO in the inflamed WM of EAE mice[69]. Under normal conditions, TSPO is expressed only at low levels in the GM in humans[69, 70]. In MS patients, significant differences in the uptake of a radioligand for TSPO were identified in specific regions of cortical GM areas compared to healthy controls; a recent study from Singhal et al. detected an increase in radioligand uptake in the hippocampus,

amygdala, posterior cingulate, midcingulate and rolandic operculum, indicating higher levels of microglial activation in these GM areas compared to healthy controls (Figure 2). However, microglia activation in total cortical GM did not differ from healthy controls[67]. A similar approach was used by Datta and colleagues. Datta et al. detected that radioligand uptake predominantly occurred in the thalamus and that MS patients showed higher radioligand binding to TSPO in this region than healthy controls. Furthermore, an increase in radioligand uptake was identified in NAWM, compared to healthy WM[71], which is supported by van Wageningen et al. who observed a decrease in the homeostatic gene expression of *P2YR12* and *TMEM119* in microglia, suggesting a shift towards reactive microglia[23].

All things considered, it is important to keep in mind that microglia are heterogeneous in both WM and GM areas. Throughout the CNS, microglia show differences in density, morphology and transcriptomic signature, with the latter being described more in detail in chapter 3. In MS, higher levels of microglia activation can be found in a number of specific GM areas, while microglia activation levels are more or less homogeneous throughout the WM. Based on their gene expression profile, these human microglia subcluster into different microglial types, while in other species microglia subcluster into only one microglial type. Moreover, male human microglia appeared to be more mature than female microglia, while interestingly this is the other way around in mice. These male mice microglia have a more primed phenotype and a higher antigen-presenting potential than female mice microglia, and, when responding to immune modulatory stimuli they push their transcriptomic signature to a more mature state. However, the question why some brain areas show higher microglial density and/or morphology compared to other brain areas still need to be answered.

2.4 Transcriptomic profiling, an initial step towards understanding MS heterogeneity

2.4.1 Low resolution transcriptomic profiling of human MS tissue

Transcriptomic profiling of human MS post-mortem brain tissues initially was performed using microarrays and bulk RNA sequencing approaches, which mainly resulted in information about neuronal cells, simply because neurons are more abundant than glial cells[72]. Therefore, combinations with laser capture microdissection (LCM), fluorescent activated cell sorting (FACS) and region-specific analysis were applied to decrease sample heterogeneity. These studies identified gene signatures of active lesions (immune related genes), which differ from the inactive lesion signature (apoptosis, stress related genes)[73, 74]. An LCM-microarray study of chronic MS lesions detected increased expression of genes encoding for heat-shock proteins (HSP) in a region dependent manner. Differences in specific HSP gene expression were observed between the margin and the center of the lesions, compared to NAWM. This lesion margin and center heterogeneity is potentially regulated by heat shock factor four (HSF4)[75]. In addition, inactive lesions increased expression of genes related to extracellular matrix components and steroid metabolism, while the rim of active lesions expressed genes related to lipid metabolism and lysosome signaling, including genes encoding for scavenger receptors (OLR1, CD68, MSR1, CXCL16), which are potential mediators of early demyelination (Figure 3)[76].

The role of lipid metabolism in early MS pathogenesis has been further elucidated, where lipid metabolism related genes (*LPL, EEPD1, CHI3L1*) were enriched in NAWM compared to CWM[4]. Besides lesion specific profiles, a region specific gene signature of the choroid plexus was identified, characterized by increased expression of hypoxia-related, neuroprotective and

secretory genes[77]. Taken together, high molecular complexity is detected between lesion types, where the immune system and lipid metabolism are major processes linked with active lesions and apoptosis, while stress response and extracellular matrix changes are related to inactive lesions.

2.4.2 High-resolution transcriptomic profiling

Bulk transcriptomic profiling contributed to MS research, although, the resolution of these technologies is limited to the analysis of pools of cells, obscuring cellular heterogeneity. Currently, a broad spectrum of sequencing technologies is available to analyze single cells (scRNAseq, see figure 3 for an overview). Two types of amplification strategies can be distinguished. First, protocols that measure full length cDNA molecules (SMART-seq2) which are most sensitive[78]. Second, protocols that measure the 3' or 5' end of cDNA molecules linked to a unique molecular identifier (UMI) (MARS-seq, STRT, CEL-seq2, Dropseq and inDrops), with highest throughput. Combinations with microfluidics and droplet-based sequencing platforms (Fluidigm C1, Chromium-10X genomics and Indrop-Cellbio) created easy to use library preparation protocols. The advantages and disadvantages of these protocols are reviewed by[79]. For human neuronal tissue, it is logistically challenging to obtain a viable single cell population

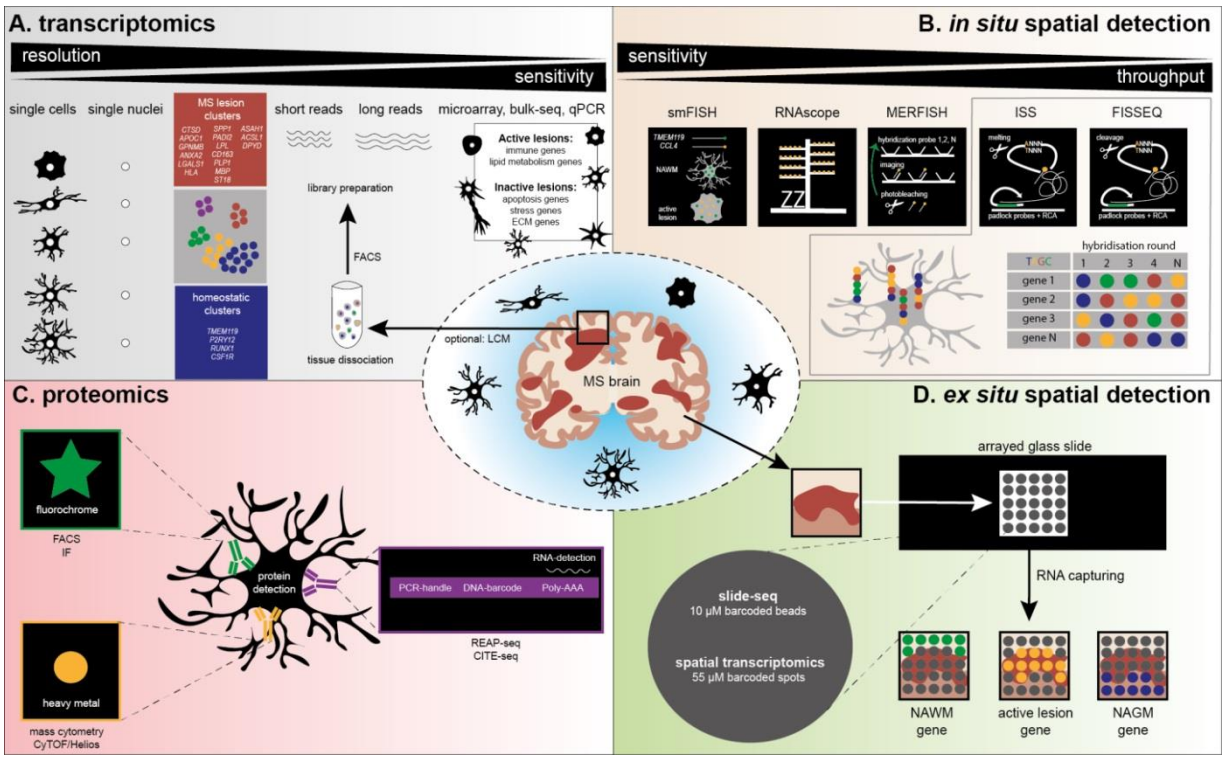

Figure 3: Illustration depicts various methodologies to detect microglia heterogeneity, (A) distinguished in transcriptomics, (B) *in situ* spatial detection, (C) proteomics, and (D) *ex situ* spatial detection. (A) After tissue dissociation and often FACS analysis, a library can be prepared to detect microglia heterogeneity. Sequencing detects heterogeneity at high (single cell/nucleus) resolution, but sensitivity is low, compared to microarray, bulk-seq, and qPCR. Several studies already detected homeostatic and MS-associated microglia clusters, characterized by the genes depicted in the blue and red squares, respectively. (B) The golden standard methods using a single probe (smFISH) and a probe with amplifiers (RNAscope) are followed by high-throughput *in situ* detection of genes, via multiple rounds of probe hybridization, cleavage, imaging, and finally sequence decoding. (C) Standardly applied proteomic methods use antibodies containing fluorophores to detect a protein, while high-throughput proteomics methodologies make use of antibodies labeled with heavy metals (mass cytometry) or DNA barcodes (REAP-seq/CITE-seq). REAP-seq and CITE-seq antibodies also contain an RNA binding site for simultaneous RNA detection. (D) A tissue section is placed on a barcoded slide with beads (slide-seq) or spots (spatial transcriptomics), which allows to explore regional gene expression in MS lesions.

from fresh tissue prior to sequencing, therefore some alternatives can be considered.

If a sufficient number of cells can be obtained, possibilities exist to use a fixation buffer such as methanol, allowing temporal storage of single cell suspensions at -80 degrees. However, not all cells will recover from this process and it should be tested if these buffers are suitable for glia cells[43, 79]. Instead of analyzing cells, individual nuclei isolated from fresh or frozen tissue, can be profiled; a technique referred to as single nucleus RNA sequencing (snRNAseq)[80]. The nuclear transcriptome of microglia has been reported to reflect the cellular transcriptome under homeostatic conditions and also following a LPS challenge[81].

2.4.2.1 Microglia subtypes

Recently, high-resolution transcriptomic profiling (Figure 3) confirmed the existence of molecularly distinct microglia subtypes, with potentially specific functionalities[10, 17, 19, 20, 83–85]. To date, dynamics of microglial subtypes have been studied under homeostasis and during development, aging, and various neurodegenerative disease conditions in human and mouse models (including cuprizone and EAE, (de)(re)myelination and inflammation model for MS respectively) (reviewed by[18]). Complexity in microglial subtypes is increased in disease compared to homeostatic conditions and is highly influenced by the brain microenvironment. scRNAseq/snRNAseq studies of microglia derived from human post-mortem brain tissue from MS donors are scarce and only available with a limited number of donors and cells. An overview of these transcriptomics studies is given in chapter 1: microglia in MS.

2.5 Spatial detection could provide new insights in regional heterogeneity

High-resolution transcriptomics technologies have made it possible to study the entire transcriptome at the level of an individual cell. These methods are highly relevant for understanding MS pathogenesis as they can detect differentially expressed genes in the affected tissue and potentially identify novel cell subtypes. However, these technologies require disruption of the tissue for the isolation of cells or nuclei from the tissue, and as a consequence spatial context is lost. However, spatial context is important in understanding cell functioning in health and during pathology. Thus, techniques that allow spatial detection could provide added value when studying (regional) heterogeneity to further elucidate pathogenic features (figure 3).

2.5.1 In situ hybridization assays

In 1969, in situ hybridization (ISH) was pioneered by Pardue et al., and this was followed by the invention of fluorescent probes around ten years later[86, 87]. (F)ISH is a technique that uses labeled DNA or RNA probes to target a specific DNA or RNA sequence within a histological section, thus indicating gene expression at a spatial level. A problem that arose with this technique was that single molecules could only be detected in regions with a low background signal. In 1998, single-molecule FISH (smFISH) was developed by Femino et al.[87]. smFISH includes probes labeled with five fluorochromes that target multiple regions of the transcript, resulting in a higher fluorescence signal, allowing specific detection of single molecules[87]. However, the use of probes consisting of five different fluorochromes was associated with a high risk of both false-positive and false-negative outcomes. Furthermore, these heavily labeled probes were difficult to develop[88, 89]. In 2009, Raj and colleagues optimized smFISH. The technique uses 40 probes, each coupled to a single fluorochrome, resulting in more accurate

mRNA counts[89]. Hammond and colleagues studied microglial RNA expression patterns in mice during development, in old age and after brain injury[84]. Following demyelination injury by lysolecitin (LPC) injection, this research group used smFISH to determine the organization of microglia within these demyelinated lesions. Three probes were used for smFISH to label the microglial marker Fcrls and chemokines *Ccl4* and *Cxcl10*. smFISH confirmed an increase in Fc receptor-like molecules+ (Fcrls+) microglia and showed higher *Apoe* expression after LPC injection compared to control mice. Furthermore, smFISH identified a spatial distribution of *Ccl4* and *Cxcl10* within the lesion; both chemokines were expressed only in small specific parts of the demyelinated area, and in most of the cases the chemokine expression colocalized with FCRLS+ microglia. In active MS lesions, also an increase in *CCL4* expression was observed, mostly in TMEM119+ microglia (Figure 3)[84]. Another study investigated regional GM heterogeneity by studying neuronal gene expression in the different layers of the cortex, to further elucidate MS pathology[20]. Using smFISH, probes for simultaneous detection of 2-3 genes of interest were included and gene expression was measured in cortical GM, adjacent subcortical WM lesion areas, and controls. smFISH showed a decrease in upper-layer neuronal marker *CUX2* in completely and incompletely demyelinated cortical MS lesions, while expression of the interneuronal marker *VIP* was maintained. Furthermore, smFISH confirmed the increase of the neuronal cell stress markers *PPIA* and *NORAD*, which were mainly upregulated in demyelinated cortical MS lesions[20]. A similar approach to smFISH is RNAscope. RNAscope uses target-specific double Z probes that target the mRNA. Then, amplifiers and different labels (chromogenic or fluorescent) can be added to these Z probes. Targeted mRNA transcripts are detected as dots, each dot representing a single copy of the mRNA. Compared to smFISH, RNAscope probes amplify signals while simultaneously suppressing the background noise[90].

In (sm)FISH, the number of fluorescent probes is generally limited to three due to limitations of fluorescent microscopy, such as the use of band- and long-pass filters[91]. Multiplexed error-robust FISH (MERFISH), a technique that was developed in 2015 allows for the detection of multiple probes. MERFISH uses multiple rounds of hybridization and imaging, with a different probe for each RNA molecule (Figure 3)[92]. These probes label the RNA with a specific combination of readout sequences, resulting in 'barcodes' assigned to the RNAs of interest. The barcodes are then read out by the use of fluorescent probes that target the RNA sequences. The higher the number of rounds, the higher the copy number of genes that can be measured simultaneously. To adjust for detection errors that exponentially increase with the number of rounds, error-robust encoding schemes were added that detect and correct errors to improve accuracy and sensitivity[92-93]. Recently smFISH was used to confirm the transcriptomics data in astrocytes from EAE mice, where *MAFG* expression was increased, while *NRF2* levels were decreased compared to control mice. In addition, MERFISH revealed that these Mafg+ astrocytes were in close proximity to granulocyte-macrophage colony-stimulating factor Gm-csf+ T cells[94]. Thus, MERFISH adds value when spatially investigating the (co-) expression of a high number of RNAs of interest simultaneously.

2.5.2 Spatial in situ sequencing techniques

Fluorescent in situ sequencing (FISSEQ) is a method that was first described by Mitra et al. in 2003. First of all, a library with molecules of linear DNA is generated. Each of these molecules contains one variable region that is linked to two constant regions. These constant regions allow primer binding during an amplification step. In-gel amplification of this library results in so-called polonies (polymerase colonies). During FISSEQ, the polonies are denatured and

sequencing primers hybridize to the template (Figure 3). Next, sequencing is performed by different cycles in which single fluorescent nucleotides are added and imaged[95]. In 2014, the next-generation of FISSEQ was developed[96-97]. This technique allows the use of both fresh-frozen (FF) tissue and formalin-fixed paraffin-embedded (FFPE) tissue. First, the fixed tissue is tagged with random hexamers, followed by reverse transcription and cDNA amplification. Next, these cDNA amplicons are cross-linked in situ and sequenced. Each base is visualized in one color, resulting in certain barcodes that can be mapped to the genome. There is no need to select candidate genes as this method captures all of the mRNA and thus can localize the RNA whole transcriptome. Other advantages of this technology are high sensitivity and high throughput. Furthermore, FISSEQ can be applied to intact cells and tissues, avoiding sectioning. However, low-abundant targets are not detected by FISSEQ, as the mRNA to cDNA conversion efficiency is limited[97-96].

In situ sequencing (ISS) was first described in 2013[98]. The ISS procedure starts with reverse transcription of mRNA to cDNA, followed by the hybridization of padlock probes to the cDNA strand (Figure 3). Two approaches were developed; gap-targeted sequencing and barcode-targeted sequencing. Both approaches are followed by rolling-circle amplification and sequencing-by-ligation, which makes it possible to sequence small RNA fragments, within cells and tissue sections. For gap-targeted sequencing one extra DNA polymerase step has to be taken to fill the gap. The barcode-targeted sequencing approach uses a padlock probe containing a barcode. During sequencing-by-ligation, anchor primers are hybridized next to the targeted sequence. Then, sequencing probes containing a fluorescent label for one specific nucleotide bind to the anchor primers, allowing the decoding of the gap-sequence or the barcode[98]. In 2017, CARTANA commercialized ISS (barcode-targeted sequencing approach) promising the simultaneous detection of 600 target genes.

2.5.3 Spatial ex situ sequencing techniques

Another approach to profile gene expression while retaining spatial tissue information is spatial transcriptomics (ST). ST was first reported by Ståhl et al. in 2016[99] and was improved by 10X Genomics in 2019 under the name '10X Visium' with an increase in resolution. ST and 10X Visium make use of special glass slides containing barcoded mRNA-capturing probes printed in spots (Figure 3). All probes within one spot have the same spatial barcode. First, samples are fixed on the glass slide followed by haematoxylin and eosin (HE) staining or immunofluorescent staining and imaging. Next, the tissue is permeabilized which allows RNA binding to the barcoded mRNA-capturing probes. This barcode will be incorporated into the cDNA during reverse transcription. The last step is library preparation and sequencing[100]. ST and 10X Visium thus combine histology and transcriptomics. An advantage of these techniques is that prior knowledge of certain genes is not needed, as the 200 million probes within a spot capture all of the mRNA in a tissue section. However, the resolution does not yet approach the single-cell level; the spots have a diameter of 100 μm (ST) or 55 μm (10X Visium) and thus multiple cells (depending on the tissue type) are captured within each spot. The use of ST could be of interest when analyzing different lesion types in MS. Several studies already analyzed gene expression profiles of different MS lesions, for example by bulk-RNA sequencing or single-cell sequencing[20, 73, 74]. ST/10X Visium retains spatial information by staining and imaging the tissue prior to tissue permeabilization. The spatial barcode makes it possible to map gene expression patterns back to their original location within the tissue section (Figure 3). ST/10X Visium thus could add insights in spatial gene expression profiles within the lesions itself or where the lesion

transitions to NAWM. A limitation of ST technology is that it is not suitable for detection of low abundant targets, which is true for the majority of microglial genes. Furthermore, only a relatively small tissue section can be analyzed, as the mRNA-capturing probes lay in squares of around 6 by 6mm (ST) or 8 by 8mm (10X Visium)[100].

A similar approach to ST/10X Visium is slide-seq, which was developed in 2019. Slide-seq also uses glass slides containing barcoded beads (Figure 3). These barcodes refer to a position on the glass slide, which makes it possible to map gene expression back to the imaged tissue section. However, in contrast to ST/10X Visium, adjacent tissue sections are used for staining and imaging, which could decrease the specificity when including heterogeneous tissues. A major advantage of slide-seq is that the barcoded beads have a resolution of 10 μm and can detect gene expression with approximate single-cell resolution[88, 101]. Furthermore, it takes about 3 hours to process the tissue, while this is around 8 hours for 10X Visium (for both techniques this excludes imaging and quality control time)[101]. However, slide-seq is not yet commercially available.

In summary, in the last few decades different techniques have been developed that make use of spatial gene expression analysis. Methods that allow for spatial detection could provide information about heterogeneity of cells and genes within tissue sections, interactions between cells, and their function and cellular composition. These findings could contribute to novel insights in the underlying mechanisms of pathologies, which are crucial in the process to identify novel therapeutic targets. Depending on the study and its aim, it is important to keep in mind the advantages and disadvantages of the spatial technologies as sensitivity, resolution and throughput differ between the spatial assays and could lead to wrong interpretation of research outcomes.

2.6 Pre-analytical factors to consider during microglia transcriptomics profiling

Both high-resolution transcriptomics and spatial detection methods measure RNA levels. In addition, high-resolution transcriptomics requires isolation of microglial cells. It remains largely unclear how pre-analytical factors such as RNA quality, post-mortem delay (PMD) and microglia isolation methods exactly influence the microglial transcriptome. These pre-analytical factors vary between labs and standard procedures are lacking. In order to obtain a pure microglia cell population, mechanical or enzymatic tissue dissociation methods are required, followed by FACS or magnetic bead sorting. Alternatively, in mice, microglia specific RNA molecules can be captured using RiboTag or PAPERCLIP technologies[102, 103]. Analysis of microglia gene expression by RiboTag identified that changes can occur upon microglia isolation, where pro-inflammatory genes are significantly upregulated due to dissociation at 37 °C[102]. The PAPERCLIP technology has the advantage that microglia cells represent a more native state, because alternative polyadenylation does not affect microglial gene expression profiles[103]. Furthermore, microglia can be obtained via LCM guided single cell isolation. However, the microglia transcriptome appeared to be different when comparing LCM-isolated and FACS-isolated microglia, where LCM-isolated microglia are not a pure microglial population, since 50 percent neuronal and oligodendroglial transcripts were also detected[104]. In mice, tissue dissociation steps can be skipped using the genetic cTag-PAPERCLIP or Ribotag model for microglia isolation, which more accurately reflects the microglia transcriptome[103]. Of note, spatial detection methodologies are always independent of microglia isolation procedures, therefore reflecting *in vivo* microglia

more accurately, of course the effect of PMD still remains. The Netherlands Brain Bank isolated microglia cells of 100 donors, including MS donors who in general have a longer PMD compared to controls, since an extended autopsy protocol was applied for MS donors using MRI to select lesions. RNA isolation and qPCR was performed on microglial cells isolated from these donors. Surprisingly, there was no effect of PMD on microglial cell yield, while cerebrospinal fluid PH had a positive correlation with the yield[105]. Next, a high RNA integrity number, reflecting no RNA degradation within the tissues, is an important factor to generate reliable and reproducible gene expression datasets. In brain tissue, RNA quality of controls has been reported to be significantly higher than in disease conditions, but, overall RNA quality was independent of PMD up to 36 hours[106]. In addition, RNA quality is independent of storage time[106, 107]. RNA quality differences within one dataset might mask biologically relevant information. Computationally it is possible to correct for RNA quality using a linear model framework[108]. However, tissue selection based on RNA quality is an important step prior to downstream analysis. Our advice is to follow RNA integrity guidelines as stated in the protocol of the company, or, if not specified, RIN ≥ 5. To summarize, RNA quality and microglia isolation procedures have larger effects on gene expression than the PMD. However, only a minimal number of studies investigated the effect of PMD on gene expression, so far these studies did not used high throughput gene expression technologies.

2.7 Proteomic studies to decode cellular heterogeneity

Proteomics is the study of the entire proteome in a cell, tissue or organism. The proteome is dynamic; modifications occur consistently by post-translational modifications, protein-protein interactions, synthesis and degradation[109, 110]. Proteins are composed of amino acids, which determine the structural properties and thus the cellular function of the proteins. Techniques to analyze specific proteins are western blotting and enzyme-linked immunosorbent assay (ELISA), however these allow the analysis of just a few proteins at a time[109]. In 1941, Coons et al. were the first to use IHC in their study for the spatial detection of protein epitopes within a tissue section[111]. Already in the eighties, HLA-DR staining was performed to study antigen representation in NAWM from MS patients compared to controls[112]. IHC is a technique that is still used, however, over the years new methods have been developed for protein detection that allow a higher throughput or are combined with transcriptomics.

One protein detection technique with a higher throughput is mass cytometry. This technique is a combination of flow cytometry and mass spectrometry. Instruments to measure mass cytometry are cytometry by time-of-flight (CyTOF), CyTOF2 and the most recent one, Helios. Both sensitivity and throughput increased with each generation of instruments. Mass cytometry requires single cell suspensions, which are incubated in a pool of antibodies conjugated to a unique, stable heavy-metal isotope (Figure 3). These isotopes are used as reporters, capable of measuring the gene expression of specific targets. Next, nebulized droplets are introduced into the inductively-coupled argon plasma (ICP). Here, ions are liberated, and cells are atomized. Ions are filtered in the quadripole, which allows only the heavy-metal reporter ions to be distinguished by their variation in mass and to be quantified by CyTOF(2) or Helios. Up to 45 parameters can be detected, which is a large improvement compared to the 8-12 parameters that can be detected by flow-cytometry[113, 114]. Mass cytometry does not only allow for the detection of proteins and their expression levels, also post-translational modifications and proteolytic products can be determined. For example, the technique is able to measure phosphorylated (activated) states of proteins, which could provide insights in cellular

behavior[114]. Mass cytometry thus contributes to the decoding of cellular heterogeneity. Ajami et al. used cell cytometry to characterize myeloid populations in EAE and other models for neurodegenerative diseases (Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS))[115]. Using CyTOF, Ajami and colleagues identified two CD11b+ myeloid populations in the healthy brain, while an extra CD11b+ myeloid population was detected in the CNS of EAE mice and in HD and ALS models. The first population (population A) was characterized by a CD317+MHC-II–CD39lowCD86– profile; the second population (population B) was CD317+MHC-II–CD39hiCD86+ and the third (population C) was CD317+MHC-II+CD39hiCD86+, indicating that populations B and C 58 consisted of activated microglia. Furthermore, CD11c was only expressed by population C. During EAE progression, population C expanded, while in chronic EAE and in the recovery phase a decrease in the number of cells in population C was observed. CyTOF revealed co-expression of GM-CSF and TNFα in most of the cells in population C during EAE onset and peak of disease, while in HD and ALS models the percentage of cells expressing these cytokines was low or absent. Another interesting observation was an increase in the expression of the signaling molecules pCREB and pMAPKAPK2 in populations B and C during the onset and subsequently during the peak of EAE. In addition, CyTOF revealed that CD49d (α4 integrin) and CD49a (α5 integrin) were only expressed on peripheral monocyte populations and not on CNS resident myeloid populations. Blocking CD49a expression attenuated EAE, highlighting the potential of CD49a as a therapeutic target[115]. This study confirms that mass cytometry is an interesting method to consider when studying cellular heterogeneity, as it is a single cell method that can distinguish different populations based on (co-) expression levels of transcription factors, signaling molecules, cytokines, and other proteins in a specific cell type. Mass cytometry has a throughput of around thousand cells per seconds, which is much lower compared to flow cytometry, making the technique time-consuming. Furthermore, as cells are ionized and atomized during mass cytometry and thus fully shattered, it is not possible to sort cells and collect them for further analysis[114, 116].

New methods, such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) and RNA expression and protein sequencing assay (REAP-seq), have been developed that measure both cell-surface protein- and gene expression levels in the same individual cell (Figure 3). Both techniques use DNA-barcoded antibodies to tag proteins of interest. These barcodes are unique oligonucleotides that contain an amplification primer and sequencing handle. After cell lysis, reverse transcription and pre-amplifications steps, library preparation can be performed followed by single-cell sequencing[117, 118]. The difference between the two techniques is the way the antibody is conjugated to the DNA sequence. In CITE-seq, antibodies are conjugated to streptavidin, which is noncovalently bound to the oligonucleotide, while in REAP-seq the antibody and oligonucleotide are conjugated by covalent bonds[117, 119]. At the moment, both techniques are not able to detect intracellular proteins, however, due to the conjugation of antibody and barcode by covalent bonds, REAP-seq has potential for intracellular labeling of proteins in future[117]. Up to 82 antibodies can be multiplexed at the same time, though, it is likely that this number will increase as CITE-seq and REAP-seq are not obstructed by signal collision as is the case in flow and mass cytometry[118].

Taken together, proteomic studies are of importance when investigating underlying mechanisms of diseases, as mRNA gene expression levels do not always reflect protein expression levels due to post-translational modifications, protein-trafficking, localizations and protein-protein interactions. Mass cytometry could provide insights as it is able to determine post-translational

modification and proteolysis products. On the other hand, since REAP-seq and CITE-seq can measure cell-surface protein- and gene expression levels in parallel, they could also contribute to a better understanding of cell functioning and/or cellular heterogeneity.

2.8 Discussion: Comparison of transcriptomics, spatial detection and proteomic methodologies. Which method should I use?

Wide availability of (novel) technologies makes it challenging to choose the most appropriate method to apply to answer specific scientific questions. Here, we provide an overview of important properties per technology, which can facilitate the decision making process (table 1) (supplementary figure 1). All sequencing-based technologies are unbiased, facilitating novel discovery, since there is no need to preselect candidate genes.

High sensitivity is a major advantage of low-resolution transcriptomics. Increasing resolution and throughput generally decreases sensitivity (20%) due to a lower library complexity, which is a major drawback of high-resolution transcriptomics and only allows the detection of variations in the most abundantly expressed genes. Sequencing methodologies differ in sequencing of whole transcripts (long reads) or sequencing of a small part of the transcript: the three or 5 prime end (short read). Short read sequencing is not suitable to detect splicing variants and has a higher error rate, making annotating genes to the genome more complex compared to long read sequencing. Therefore, long read sequencing is more sensitive compared to short read sequencing. However, in terms of examining heterogeneity and identifying microglia subtypes, high-resolution is absolutely required. High-resolution transcriptomics is possible at the single cell or single nucleus level. snRNAseq has some advantages above scRNAseq; first, nuclear RNA molecules are less vulnerable to stress and can be obtained from frozen archived tissue samples, which can be very precisely stratified and classified for example into MS lesion types with matching HLA-DR scores. Nuclear RNA represents a more reliable ratio of cell subtypes since some subtypes are more affected by isolation procedures. Secondly, nuclear RNA molecules reflect active transcription which might result in a more accurate view of the condition of interest. snRNAseq of microglia or other less abundant cell types benefits from enrichment strategies to obtain sufficient numbers for in-depth analysis. So far, studies have used a direct microglial enrichment strategy based on IRF8 sorting[4] or an indirect strategy by selecting a NEUN/OLIG2 negative population to get rid of major cell types of the brain: neurons and oligodendrocytes[81]. Both single cell- and single nuclei sequencing require extensive validation experiments to prove the existence and functionality of the detected subtypes, since spatial information is lacking.

Cell shape and localization often reflect functionality, which is already an old concept in biology. Therefore, spatial detection methods offer great possibilities to examine heterogeneity and functionality at the same time in a region specific manner, by using ex situ (spatial transcriptomics, slide-seq) or in situ (FISSEQ, MERFISH, ISS) technologies, reviewed here[120, 121]. However, can we obtain similar resolution and throughput as in single cell/nuclei sequencing? Not at the moment. The resolution of ex situ spatial detection is 55 μM (1-10 cells/spot) for a commercially available method from 10X Genomics (Visium), as resolution is limited by printing distinguishable barcodes[99, 100, 122].

That level of resolution could cause problems if your goal is to determine the contribution of the microglial transcriptome for a specific condition or disease, since microglia compromise only

10% of all brain cell types and since specific microglial subtypes are expected to represent an even smaller percentage. Compared to astrocyte and oligodendrocyte populations these subsets likely provide too few RNA molecules to be detected and visualized in spatial transcriptomics in the mouse brain (figure 4). Solving this issue would require higher resolution, higher sensitivity and better computational deconvolution strategies. In comparison, slide-seq uses barcoded beads to increase the resolution to 10 µM (1-2 cells/bead) and detection of genes is unbiased, although standardized description of the protocol is not available yet[101]. Throughput of all spatial detection methodologies is limited by imaging speed, therefore measuring the complete tissue section is often not possible or very expensive. In situ spatial detection methods are very suitable for regional validation of target genes in a cell specific manner. ISS by CARTANA[98, 123] is a very promising and easy to use methodology, where individual genes are targeted by barcoded padlock probes, which is a more sensitive methodology compared to unbiased in situ approaches including FISSEQ[96]. More specifically, the sensitivity of ST (7%), ISS (10%), FISSEQ (0,005%) are all lower compared to single cell or nuclei sequencing (20%). Other disadvantages of ISS are limited throughput and limited detected targets (up to 90 genes) restricted to predetermined target genes and the design of novel padlock probes is costly. However, the next generation ISS allows detection of up to 600 genes and does not use padlock probes.

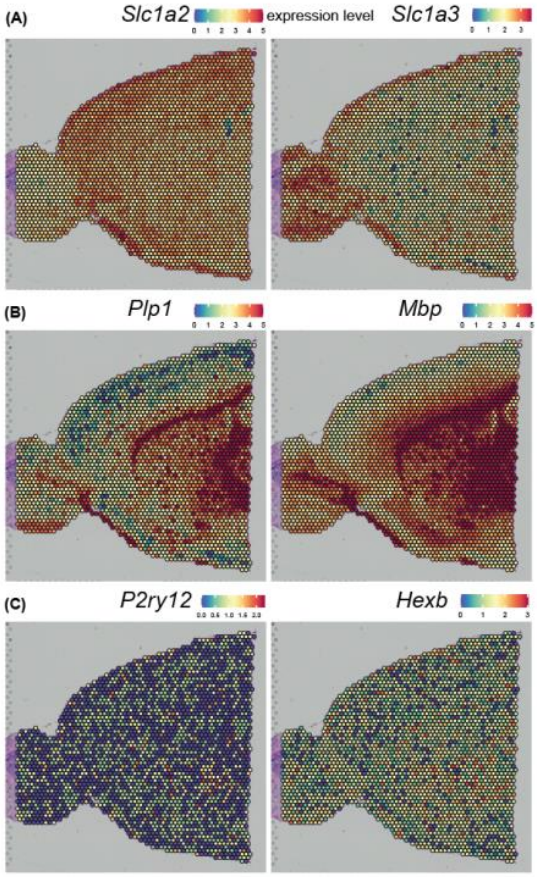

Figure 4: Spatial gene expression of astrocyte (A), oligodendrocytes (B) and microglia (C) cell specific marker genes, visualized in sagittal anterior control mouse brain. Dataset obtained from: 10X Genomics.

Proteomics technologies have some advantages above transcriptomic technologies. Proteomics is generally easier to apply, because proteins are more stable than RNA molecules, though this might not be true for all post-translational modifications. Most proteomics technologies are also cheaper compared to transcriptomics, however, exceptions are for example CyTOF which requires the production of many antibodies. mRNA expression does not always predict protein expression levels in a cell. Therefore, proteomics could contribute to important insights in cell functioning. This will be a major advantage when the study goal is to find new effective drugs, since most therapeutics target proteins and not the mRNA, which makes proteomics crucial for drug development. For example, with a proteomic strategy, Ajami et al discovered CD49a as a potential new drug target for MS[115]. Furthermore, proteins are often adapted with post-translational modifications such as phosphorylation, lipidation and glycosylation, resulting in, for example, active forms of a protein with slightly changed folding or conformation. Proteomics technologies are able to distinguish the phosphorylated (active) state of a protein, from the non-phosphorylated (inactive) state[114], which is highly important for biological data interpretation and cannot be detected with transcriptomics. High throughput proteomic methodologies such as

REAP-seq and CITE-seq, can perform proteome and transcriptome analysis simultaneously, which allows to compare the levels of both molecules in one biological sample. Despite these major advantages, there are also disadvantages to this type of proteomics technique; often not all proteins can be detected easily, and some technologies can only detect extracellular and not intracellular proteins. Moreover, protocols for proteomic methodologies are not commercially available, thus requiring more skills to robustly perform these technologies. Furthermore, proteomics can often detect a lower number of targets simultaneously compared to transcriptomics, however this is expected to increase in the future.

2.9 Future perspectives

Region and cell (sub)type specific profiling as reviewed here, have promising applications in all heterogeneous diseases to investigate molecular disease drivers and facilitate drug target discovery. In addition, it can drive the field of personalized medicine towards a region and cell (sub)type specific level. Region specific pathogenic subtypes can potentially be reprogrammed into a homeostatic or more beneficial state via localized drug delivery. Moreover, creating a landscape of cell (sub)type specific datasets allows studying interactions between cell (sub)types, for example ligand receptor mapping[124]. Furthermore, it can help to identify the drivers behind spatial heterogeneity; is this solely determined by the environment or do cell intrinsic mechanisms also play a role? In the context of MS, this information can be linked with genome wide association studies (GWAS), which provide information about single nucleotide polymorphisms (SNPs) associated with higher MS risk[125].

Finally, to facilitate the extraction of biologically relevant information from these large datasets, public platforms should be generated to share datasets produced by different research groups, stratified by disease, region and cell (sub)types. Three existing RNA sequencing databases are GOAD – now Brain-sat[126], Brain RNA-seq[127] and Neuroexpresso[128], which can be used to obtain glial cell type specific information. Currently, region specific information is lacking in these databases. For analysis of distinct regions, the mouse[129] or human brain atlas[130] can be used to annotate brain regions in spatial transcriptomics datasets. The Allen Brain Atlas already extended their anatomic data with genomic data, combining microarray, ISH and MRI datasets. Future databases should extend this even further with protein and spatial information. The next step would be to integrate such a database with information about chromatin states associated with transcription regulation, such as chromatin accessibility (ATAQ-seq), particular histone marks (CHIP-seq) or interactions within the genome and chromatin conformation information (Hi-c). Recently, an MS study by Factor et al. showed that this type of analysis can result in valuable information. This study showed that the enhancer elements *BRD3* and *HEXIM1* are directly associated with an MS risk gene and that these enhancers were dysregulated in MS, contributing to remyelination failure[131]. Thus, these databases can help scientists to extend scientific knowledge and interpret their findings in future studies. Overall, recent advances in high-resolution gene expression profiling of microglia in MS contributed to the identification of MS signatures in cell subtypes; it makes researchers aware of the heterogeneity present in MS tissues and it provides promising candidate genes for future functional analysis.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

AM and MHCW wrote the manuscript and designed the figures. BJLE and SMK revised the manuscript.

Funding

AM, SMK are supported by a fellowship from the Dutch MS Research Foundation (# 16-947). MHCW is supported by a grant from the Dutch MS Research Foundation (#18-733c).

Abbreviations

ALS, amyotrophic lateral sclerosis; ATAC-seq, assay for transposase-accessible chromatin sequencing; BBB, blood–brain barrier; CC, corpus callosum; CHIP-seq, chromatin immunoprecipitation sequencing; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; CNS, central nervous system; CyTOF, cytometry by time-of-flight; EAE, experimental-autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescent-activated cell sorting; FF, fresh frozen; FPE, formalin-fixed paraffin-embedded; FISH, fluorescent in situ hybridization; FISSEQ, fluorescent in situ sequencing; GM, gray matter; GML, gray matter lesion; GO, Gene ontology; GWAS, genome-wide association study; HD, Huntington's disease; HE, hematoxylin and eosin; ICP, inductively coupled argon plasma; IHC, immunohistochemistry; ISH, in situ hybridization; ISS, in situ sequencing; LCM, laser capture microdissection; LPC, lysolecitin; LPS, lipopolysaccharide; MERFISH, multiplexed error-robust fluorescent in situ hybridization; MRI, magnetic resonance imaging; MS, multiple sclerosis; NAGM, normal-appearing gray matter; NAWM, normal-appearing white matter; OPC, oligodendrocyte precursor cell; PMD, post-mortem delay; qPCR, quantitative polymerase chain reaction; RCA, rolling circle amplification; REAPseq, RNA expression and protein sequencing assay; ROS, reactive oxygen species; RRMS, relapsing–remitting multiple sclerosis; scRNAseq, single-cell RNA sequencing; smFISH, single-molecule fluorescent in situ hybridization; SNP, single-nucleotide polymorphisms; snRNAseq, single-nucleus RNA sequencing; ST, spatial transcriptomics; TLR, toll-like receptor; UMI, unique molecular identifier; WM, white matter; WML, white matter lesion

Supplementary figures/tables

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2020.583811/full#supplementary-material

Supplementary figure

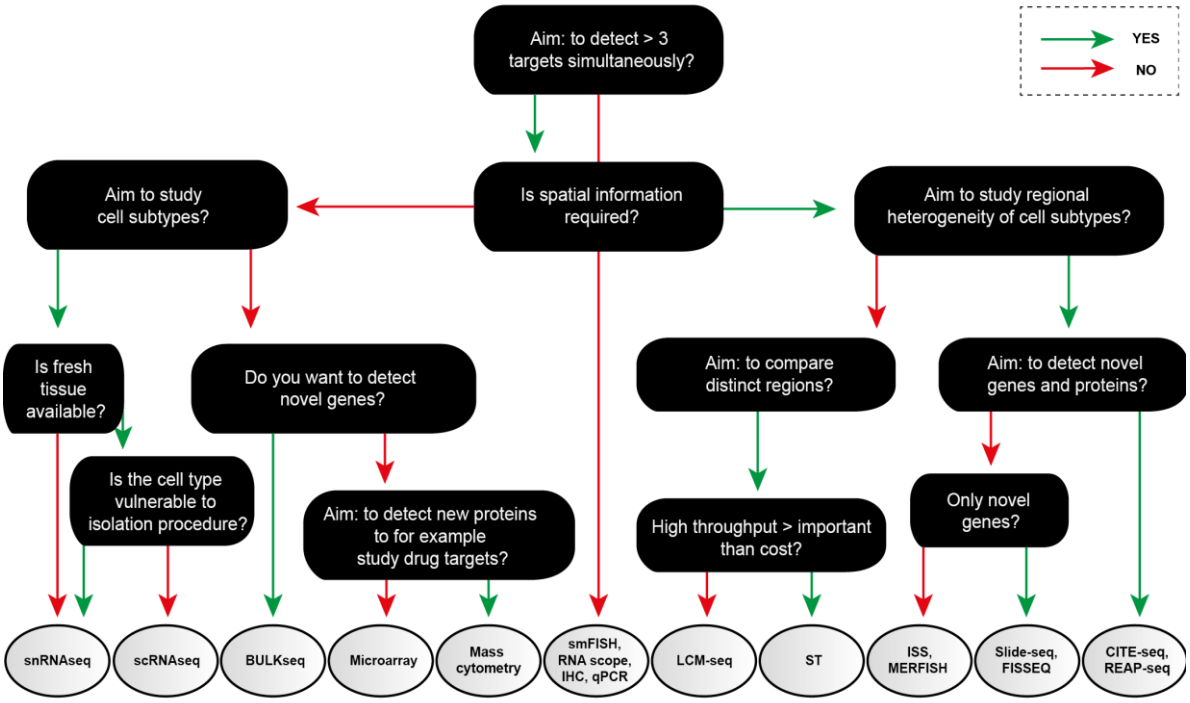

Figure S1: Decision tree, which technology is appropriate for your research aim.

2.10 References

1. Kuhlmann, T. *et al.* An updated histological classification system for multiple sclerosis lesions. *Acta Neuropathol.* **133**, 13–24 (2017).
2. Anderson, A. C. *et al.* Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. *Science (80-.).* **318**, 1141–1143 (2007).
3. McKay, S. M., Brooks, D. J., Hu, P. & McLachlan, E. M. Distinct types of microglial activation in white and grey matter of rat lumbosacral cord after mid-thoracic spinal transection. *J. Neuropathol. Exp. Neurol.* **66**, 698–710 (2007).
4. van der Poel, M. *et al.* Transcriptional profiling of human microglia reveals grey–white matter heterogeneity and multiple sclerosis-associated changes. *Nat. Commun.* **10**, 1–13 (2019).
5. Lawson, L. J., Perry, V. H., Dri, P. & Gordon, S. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience* **39**, 151–170 (1990).
6. Hanamsagar, R. *et al.* Generation of a microglial developmental index in mice and in humans reveals a sex difference in maturation and immune reactivity. *Glia* **65**, 1504–1520 (2017).
7. Guneykaya, D. *et al.* Transcriptional and Translational Differences of Microglia from Male and Female Brains. *Cell Rep.* **24**, 2773-2783.e6 (2018).
8. Villa, A. *et al.* Sex-Specific Features of Microglia from Adult Mice. *Cell Rep.* **23**, 3501–3511 (2018).
9. Lenz, K. M., Nugent, B. M., Haliyur, R. & McCarthy, M. M. Microglia are essential to masculinization of brain and behavior. *J. Neurosci.* **33**, 2761–2772 (2013).
10. Geirsdottir, L. *et al.* Cross-Species Single-Cell Analysis Reveals Divergence of the Primate Microglia Program. *Cell* **179**, 1609-1622.e16 (2019).
11. Torres-Platas, S. G. *et al.* Morphometric characterization of microglial phenotypes in human cerebral cortex. *J. Neuroinflammation* **11**, 1–13 (2014).
12. Gosselin, D. *et al.* An environment-dependent transcriptional network specifies human microglia identity. *Science (80-.).* **356**, 1248–1259 (2017).
13. Galatro, T. F. *et al.* Transcriptomic analysis of purified human cortical microglia reveals age-associated changes. *Nat. Neurosci.* **20**, 1162–1171 (2017).
14. Grabert, K. *et al.* Microglial brain regionâ 'dependent diversity and selective regional sensitivities to aging. *Nat. Neurosci.* **19**, 504–516 (2016).
15. Brioschi, S., Peng, V. & Colonna, M. Fifty Shades of Microglia. *Trends Neurosci.* **42**, 440–443 (2019).
16. Hammond, T. R. *et al.* Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. *Immunity* **50**, 253-271.e6 (2019).
17. Keren-Shaul, H. *et al.* A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell* **169**, 1276-1290.e17 (2017).
18. Masuda, T., Sankowski, R., Staszewski, O. & Prinz, M. Microglia Heterogeneity in the Single-Cell Era. *Cell Rep.* **30**, 1271–1281 (2020).
19. Mathys, H. *et al.* Temporal Tracking of Microglia Activation in Neurodegeneration at Single-Cell Resolution. *Cell Rep.* **21**, 366–380 (2017).
20. Schirmer, L. *et al.* Neuronal vulnerability and multilineage diversity in multiple sclerosis. *Nature* **573**, 75–82 (2019).
21. Dubbelaar, M. L., Kracht, L., Eggen, B. J. L. & Boddeke, E. W. G. M. The Kaleidoscope of Microglial Phenotypes. *Front. Immunol.* **9**, 1753 (2018).
22. Beaino, W. *et al.* Purinergic receptors P2Y12R and P2X7R: Potential targets for PET imaging of microglia phenotypes in multiple sclerosis. *J. Neuroinflammation* **14**, 1–16 (2017).
23. Van Wageningen, T. A. *et al.* Regulation of microglial TMEM119 and P2RY12 immunoreactivity in multiple sclerosis white and grey matter lesions is dependent on their inflammatory environment. *Acta Neuropathol. Commun.* **7**, 1–16 (2019).
24. Zrzavy, T. *et al.* Loss of 'homeostatic' microglia and patterns of their activation in active multiple sclerosis. *Brain* **140**, 1900–1913 (2017).
25. van Horssen, J. *et al.* Clusters of activated microglia in normal-appearing white matter show signs of innate immune activation. *J. Neuroinflammation* **9**, 1–9 (2012).
26. Pereira, L. *et al.* IL-10 regulates adult neurogenesis by modulating ERK and STAT3 activity. *Front. Cell. Neurosci.* **9**, 1–9 (2015).
27. Zhou, Z., Peng, X., Insolera, R., Fink, D. J. & Mata, M. Interleukin-10 provides direct trophic support to neurons. *J. Neurochem.* **110**, 1617–1627 (2009).
28. Grajchen, E., Hendriks, J. J. A. & Bogie, J. F. J. The physiology of foamy phagocytes in multiple sclerosis. *Acta Neuropathol. Commun.* **6**, 124 (2018).
29. Hametner, S. *et al.* Iron and neurodegeneration in the multiple sclerosis brain. *Ann. Neurol.* **74**, 848–861 (2013).
30. Hametner, S., Dal Bianco, A., Trattnig, S. & Lassmann, H. Iron related changes in MS lesions and their validity to characterize MS lesion types and dynamics with Ultra-high field magnetic resonance imaging. *Brain Pathol.* **28**, 743–749 (2018).
31. Yauger, Y. J. *et al.* Iron accentuated reactive oxygen species release by NADPH oxidase in activated microglia contributes to oxidative stress in vitro. *J. Neuroinflammation* **16**, 1–15 (2019).
32. Lee, J., Hamanaka, G., Lo, E. H. & Arai, K. Heterogeneity of microglia and their differential roles in white matter pathology. *CNS Neurosci. Ther.* **25**, 1290–1298 (2019).
33. Mairuae, N., Connor, J. R. & Cheepsunthorn, P. Increased cellular iron levels affect matrix metalloproteinase expression and phagocytosis in activated microglia. *Neurosci. Lett.* **500**, 36–40 (2011).

34. Gillen, K. M., Mubarak, M., Nguyen, T. D. & Pitt, D. Significance and in vivo detection of iron-laden microglia in white matter multiple sclerosis lesions. *Front. Immunol.* **9**, 1–8 (2018).
35. Dal-Bianco, A. *et al.* Slow expansion of multiple sclerosis iron rim lesions: pathology and 7 T magnetic resonance imaging. *Acta Neuropathol.* **133**, 25–42 (2017).
36. Miron, V. E. *et al.* M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat. Neurosci.* **16**, 1211–1218 (2013).
37. Butovsky, O. *et al.* Microglia activated by IL-4 or IFN-γ differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol. Cell. Neurosci.* **31**, 149–160 (2006).
38. Cunha, M. I. *et al.* Pro-inflammatory activation following demyelination is required for myelin clearance and oligodendrogenesis. *J. Exp. Med.* **217**, (2020).
39. Lampron, A. *et al.* Inefficient clearance of myelin debris by microglia impairs remyelinating processes. *J. Exp. Med.* **212**, 481–495 (2015).
40. Petiet, A., Aigrot, M. S. & Stankoff, B. Gray and white matter demyelination and remyelination detected with multimodal quantitative mri analysis at 11.7T in a chronic mouse model of multiple sclerosis. *Front. Neurosci.* **10**, 1–10 (2016).
41. Luchetti, S. *et al.* Progressive multiple sclerosis patients show substantial lesion activity that correlates with clinical disease severity and sex: a retrospective autopsy cohort analysis. *Acta Neuropathol.* **135**, 511–528 (2018).
42. Howell, O. W. *et al.* Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain* **134**, 2755–2771 (2011).
43. Böttcher, C. *et al.* Human microglia regional heterogeneity and phenotypes determined by multiplexed single-cell mass cytometry. *Nat. Neurosci.* **22**, 78–90 (2019).
44. de Haas, A. H., Boddeke, H. W. G. M. & Biber, K. Region-specific expression of immunoregulatory proteins on microglia in the healthy CNS. *Glia* **56**, 888–894 (2008).
45. Mittelbronn, M., Dietz, K., Schluesener, H. J. & Meyermann, R. Local distribution of microglia in the normal adult human central nervous system differs by up to one order of magnitude. *Acta Neuropathol.* **101**, 249–255 (2001).
46. Savchenko, V. L., Nikonenko, I. R., Skibo, G. G. & McKanna, J. A. Distribution of microglia and astrocytes in different regions of the normal adult rat brain. *Neurophysiology* **29**, 343–351 (1997).
47. Kondo, Y. & Duncan, I. D. Selective reduction in microglia density and function in the white matter of colony-stimulating factor-1-deficient mice. *J. Neurosci. Res.* **87**, 2686–2695 (2009).
48. Cătălin, B., Mitran, S., Albu, C. & Iancău, M. Comparative aspects of microglia reaction in white and gray matter. *Curr. Heal. Sci. J.* **39**, 151–4 (2013).
49. Verdonk, F. *et al.* Phenotypic clustering: A novel method for microglial morphology analysis. *J. Neuroinflammation* **13**, (2016).
50. Skripuletz, T. *et al.* Cortical demyelination is prominent in the murine cuprizone model and is strain-dependent. *Am. J. Pathol.* **172**, 1053–1061 (2008).
51. Gudi, V. *et al.* Regional differences between grey and white matter in cuprizone induced demyelination. *Brain Res.* **1283**, 127–138 (2009).
52. Tomassy, G. S. *et al.* Distinct profiles of myelin. **344**, 319–324 (2014).
53. Chang, A. *et al.* Cortical remyelination: A new target for repair therapies in multiple sclerosis. *Ann. Neurol.* **72**, 918–926 (2012).
54. Gingele, S. *et al.* Delayed Demyelination and Impaired Remyelination in Aged Mice in the Cuprizone Model. *Cells* **9**, (2020).
55. Constantinescu, C. S., Farooqi, N., O'Brien, K. & Gran, B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br. J. Pharmacol.* **164**, 1079–1106 (2011).
56. Torkildsen, Brunborg, L. A., Myhr, K. M. & Bø, L. The cuprizone model for demyelination. *Acta Neurol. Scand.* **117**, 72–76 (2008).
57. Torres-Platas, G, S Comeau, S. *et al.* Morphometric characterization of microglial phenotypes in human cerebral cortex. *J. Neuroinflammation* **11:12**, (2014).
58. Geirsdottir, L. *et al.* Cross-Species Single-Cell Analysis Reveals Divergence of the Primate Microglia Program. *Cell* **179**, 1609-1622.e16 (2019).
59. Holtman, I. R. *et al.* Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: a co-expression meta-analysis. *Acta Neuropathol. Commun.* **3**, 31 (2015).
60. Hayflick, L. The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* **37**, 614–636 (1965).
61. Askew, K. *et al.* Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain. *Cell Rep.* **18**, 391–405 (2017).
62. Sellner, J. *et al.* The increasing incidence and prevalence of female multiple sclerosis-A critical analysis of potential environmental factors. *Autoimmun. Rev.* **10**, 495–502 (2011).
63. Tremlett, H. *et al.* Relapses in multiple sclerosis are age- and time-dependent. *J. Neurol. Neurosurg. Psychiatry* **79**, 1368–1374 (2008).
64. Ribbons, K. A. *et al.* Male sex is independently associated with faster disability accumulation in relapse-onset MS but not in primary progressive MS. *PLoS One* **10**, 1–11 (2015).
65. Kodama, L. & Gan, L. Do Microglial Sex Differences Contribute to Sex Differences in Neurodegenerative Diseases? *Trends Mol. Med.* **25**, 741–749 (2019).
66. Beckers, L. *et al.* Increased Expression of Translocator Protein (TSPO) Marks Pro-inflammatory Microglia but Does Not Predict Neurodegeneration. *Mol. Imaging Biol.* **20**, 94–102 (2018).
67. Singhal, T. *et al.* Gray matter microglial activation in relapsing vs progressive MS: A [F-18]PBR06-PET study. *Neurol.*

Neuroimmunol. NeuroInflammation **6**, (2019).
68. Chechneva, O. V. & Deng, W. Mitochondrial translocator protein (TSPO), astrocytes and neuroinflammation. *Neural Regen. Res.* **11**, 1056–1057 (2016).
69. Vowinckel, E. *et al.* PK11195 binding to the peripheral benzodiazepine receptor as a marker of microgila activation in multiple sclerosis and experimental autoimmune encephalomyelitis. *J. Neurosci. Res.* **50**, 345–353 (1997).
70. Banati, R. B. Visualising microglial activation in vivo. *Glia* **40**, 206–217 (2002).
71. Datta, G. *et al.* 11C-PBR28 and 18F-PBR111 detect white matter inflammatory heterogeneity in multiple sclerosis. *J. Nucl. Med.* **58**, 1477–1482 (2017).
72. Zheng, W. *et al.* Identification of key target genes and biological pathways in multiple sclerosis brains using microarray data obtained from the Gene Expression Omnibus database. *Neurol. Res.* (2018) doi:10.1080/01616412.2018.1497253.
73. Elkjaer, M. L. *et al.* Molecular signature of different lesion types in the brain white matter of patients with progressive multiple sclerosis. *Acta Neuropathol. Commun.* **7**, 1–17 (2019).
74. Mycko, M. P., Papoian, R., Boschert, U., Raine, C. S. & Selmaj, K. W. Microarray gene expression profiling of chronic active and inactive lesions in multiple sclerosis. *Clin. Neurol. Neurosurg.* **106**, 223–229 (2004).
75. Mycko, M. P., Brosnan, C. F., Raine, C. S., Fendler, W. & Selmaj, K. W. Transcriptional profiling of microdissected areas of active multiple sclerosis lesions reveals activation of heat shock protein genes. *J. Neurosci. Res.* **90**, 1941–1948 (2012).
76. Hendrickx, D. A. E. *et al.* Gene expression profiling of multiple sclerosis pathology identifies early patterns of demyelination surrounding chronic active lesions. *Front. Immunol.* **8**, (2017).
77. Rodríguez-Lorenzo, S. *et al.* Altered secretory and neuroprotective function of the choroid plexus in progressive multiple sclerosis. *Acta Neuropathol. Commun.* **8**, 1–13 (2020).
78. Picelli, S. *et al.* Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* **9**, 171–181 (2014).
79. See, P., Lum, J., Chen, J. & Ginhoux, F. A single-cell sequencing guide for immunologists. *Front. Immunol.* **9**, 1–13 (2018).
80. Krishnaswami, S. R. *et al.* Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons. *Nat. Protoc.* **11**, 499–524 (2016).
81. Gerrits, E., Heng, Y., Boddeke, E. W. G. M. & Eggen, B. J. L. Transcriptional profiling of microglia; current state of the art and future perspectives. *Glia* **68**, 740–755 (2020).
82. Masuda, T. *et al.* Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. *Nature* **566**, 388–392 (2019).
83. Jordão, M. J. C. *et al.* Neuroimmunology: Single-cell profiling identifies myeloid cell subsets with distinct fates during neuroinflammation. *Science (80-.).* **363**, (2019).
84. Hammond, T. R. *et al.* Single cell RNA sequencing of microglia throughout the mouse lifespan and in the injured brain reveals complex cell-state changes. *Immunity* **50**, 253–271 (2019).
85. Pardue, M. Lou & Gall, J. G. Molecular hybridization of radioactive DNA to the DNA of cytological preparations. *Proc. Natl. Acad. Sci.* **64**, 600–604 (1969).
86. Rudkin, G. T. & Stollar, B. D. High resolution detection of DNA–RNA hybrids in situ by indirect immunofluorescence. *Nature* **265**, 472–473 (1977).
87. Femino, A. M., Fay, F. S., Fogarty, K. & Singer, R. H. Visualization of single RNA transcripts in situ. *Science (80-.).* **280**, 585–590 (1998).
88. Asp, M., Bergenstråhle, J. & Lundeberg, J. Spatially Resolved Transcriptomes — Next Generation Tools for Tissue Exploration. **1900221**, 1–16 (2020).
89. Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A. & Tyagi, S. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat. Methods* **5**, 877–879 (2008).
90. Wang, F. *et al.* RNAscope: A novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J. Mol. Diagnostics* **14**, 22–29 (2012).
91. Lukumbuzya, M., Schmid, M., Pjevac, P. & Daims, H. A multicolor fluorescence in situ hybridization approach using an extended set of fluorophores to visualize microorganisms. *Front. Microbiol.* **10**, 1–13 (2019).
92. Chen, K. H., Boettiger, A. N., Moffitt, J. R., Wang, S. & Zhuang, X. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science (80-.).* **348**, 1360–1363 (2015).
93. Strell, C. *et al.* Placing RNA in context and space – methods for spatially resolved transcriptomics. *FEBS J.* **286**, 1468–1481 (2019).
94. Wheeler, M. A. *et al.* MAFG-driven astrocytes promote CNS inflammation. *Nature* **578**, 593–599 (2020).
95. Mitra, R. D., Shendure, J., Olejnik, J., Krzymanska-Olejnik, E. & Church, G. M. Fluorescent in situ sequencing on polymerase colonies. *Anal. Biochem.* **320**, 55–65 (2003).
96. Lee, J. H. *et al.* Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues Competing financial interests Potential conflicts of interests for. *Nat Protoc* **10**, 442–458 (2015).
97. Lee, J. H. *et al.* Highly multiplexed subcellular RNA sequencing in situ. 1360–1364 (2014).
98. Ke, R. *et al.* In situ sequencing for RNA analysis in preserved tissue and cells. *Nat. Methods* **10**, 857–860 (2013).
99. Ståhl, P. L. *et al.* Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. *Science (80-.).* **353**, 78–82 (2016).
100. Salmén, F. *et al.* Barcoded solid-phase RNA capture for Spatial Transcriptomics profiling in mammalian tissue sections. *Nat. Protoc.* **13**, 2501–2534 (2018).
101. Rodriques, S. G. *et al.* Slide-seq: A scalable technology for measuring genome-wide expression at high spatial resolution. *Science (80-.).* **363**, 1463–1467 (2019).
102. Haimon, Z. *et al.* Re-evaluating microglia expression profiles using RiboTag and cell isolation strategies /631/1647/2017

/631/1647/2017/2079 technical-report. *Nat. Immunol.* **19**, 636–644 (2018).
103. Hwang, H. W. *et al.* cTag-PAPERCLIP Reveals Alternative Polyadenylation Promotes Cell-Type Specific Protein Diversity and Shifts Araf Isoforms with Microglia Activation. *Neuron* **95**, 1334-1349.e5 (2017).
104. Solga, A. C. *et al.* RNA-sequencing reveals oligodendrocyte and neuronal transcripts in microglia relevant to central nervous system disease. *Glia* **63**, 531–548 (2015).
105. Mizee, M. R. *et al.* Isolation of primary microglia from the human post-mortem brain: effects of ante- and post-mortem variables. *Acta Neuropathol. Commun.* **5**, 16 (2017).
106. White, K. *et al.* Effect of Postmortem Interval and Years in Storage on RNA Quality of Tissue at a Repository of the NIH NeuroBioBank. *Biopreserv. Biobank.* **16**, 148–157 (2018).
107. Shah, S. G. *et al.* Establishing a correlation between RIN and A260/280 along with the multivariate evaluation of factors affecting the quality of RNA in cryopreserved cancer bio-specimen. *Cell Tissue Bank.* **20**, 489–499 (2019).
108. Gallego Romero, I., Pai, A. A., Tung, J. & Gilad, Y. RNA-seq: Impact of RNA degradation on transcript quantification. *BMC Biol.* **12**, 1–13 (2014).
109. Aslam, B., Basit, M., Nisar, M. A., Khurshid, M. & Rasool, M. H. Proteomics: Technologies and their applications. *J. Chromatogr. Sci.* **55**, 182–196 (2017).
110. Chandramouli, K. & Qian, P.-Y. Proteomics: Challenges, Techniques and Possibilities to Overcome Biological Sample Complexity. *Hum. Genomics Proteomics* **1**, (2009).
111. Coons, A. H., Creech, H. J. & Jones, R. N. Immunological Properties of an Antibody Containing a Fluorescent Group. *Proc. Soc. Exp. Biol. Med.* **47**, 200–202 (1941).
112. Hayes, G. M., Woodroofe, M. N. & Cuzner, M. L. Microglia Express MHC Class II in Normal and Demyelinating Human White Matter. *Ann. N. Y. Acad. Sci.* **540**, 501–503 (1988).
113. Bendall, S. C., Nolan, G. P., Roederer, M. & Chattopadhyay, P. K. A deep profiler's guide to cytometry. *Trends Immunol.* **33**, 323–332 (2012).
114. Spitzer, M. H. & Nolan, G. P. Mass Cytometry: Single Cells, Many Features. *Cell* **165**, 780–791 (2016).
115. Ajami, B. *et al.* Single-cell mass cytometry reveals distinct populations of brain myeloid cells in mouse neuroinflammation and neurodegeneration models. *Nat. Neurosci.* **21**, 541–551 (2018).
116. Bandura, D. R. *et al.* Mass cytometry: Technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry. *Anal. Chem.* **81**, 6813–6822 (2009).
117. Peterson, V. M. *et al.* Multiplexed quantification of proteins and transcripts in single cells. *Nat. Biotechnol.* **35**, 936–939 (2017).
118. Stoeckius, M. *et al.* Simultaneous epitope and transcriptome measurement in single cells. *Nat. Methods* **14**, 865–868 (2017).
119. Todorovic, V. Single-cell RNA-seq—now with protein. *Nat. Methods* **14**, 1028–1029 (2017).
120. Lein, E., Borm, L. E. & Linnarsson, S. The promise of spatial transcriptomics for neuroscience in the era of molecular cell typing. *Science (80-.).* **358**, 64–69 (2017).
121. Lee, J. H. Quantitative approaches for investigating the spatial context of gene expression. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **9**, 17–19 (2017).
122. Gregory, J. M. *et al.* Spatial transcriptomics identifies spatially dysregulated expression of GRM3 and USP47 in amyotrophic lateral sclerosis. *Neuropathol. Appl. Neurobiol.* **72**, 1–17 (2020).
123. Maïno, N. *et al.* A microfluidic platform towards automated multiplexed in situ sequencing. *Sci. Rep.* **9**, 1–10 (2019).
124. Cabello-Aguilar, S. *et al.* SingleCellSignalR: inference of intercellular networks from single-cell transcriptomics. *Nucleic Acids Res.* 1–12 (2020) doi:10.1093/nar/gkaa183.
125. Patsopoulos, N. A. *et al.* Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science (80-.).* **365**, (2019).
126. Holtman, I. R. *et al.* Glia Open Access Database (GOAD): A comprehensive gene expression encyclopedia of glia cells in health and disease. *Glia* **63**, 1495–1506 (2015).
127. Zhang, Y. *et al.* An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J. Neurosci.* **34**, 11929–11947 (2014).
128. Mancarci, B. O. *et al.* Cross-laboratory analysis of brain cell type transcriptomes with applications to interpretation of bulk tissue data. *eNeuro* **4**, (2017).
129. Erö, C., Gewaltig, M. O., Keller, D. & Markram, H. A cell atlas for the mouse brain. *Front. Neuroinform.* **12**, 1–16 (2018).
130. Sunkin, S. M. *et al.* Allen Brain Atlas: An integrated spatio-temporal portal for exploring the central nervous system. *Nucleic Acids Res.* **41**, (2013).
131. Factor, D. C. *et al.* Cell Type-Specific Intralocus Interactions Reveal Oligodendrocyte Mechanisms in MS Article Cell Type-Specific Intralocus Interactions Reveal Oligodendrocyte Mechanisms in MS. *Cell* 1–14 (2020) doi:10.1016/j.cell.2020.03.002.
132. Foley, J. W. *et al.* Gene expression profiling of single cells from archival tissue with laser-capture microdissection and Smart-3SEQ. *Genome Res.* **29**, 1816–1825 (2019).