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Robert Weissert *Editor*

Multiple Sclerosis

Methods and Protocols

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Preface

The book *Multiple Sclerosis: Methods and Protocols* contains 17 chapters with state-of-the-art methods and techniques contributed by eminent scientists that will enable investigators to perform research for multiple sclerosis (MS). First of all, I want to thank John M. Walker (Hertfordshire, United Kingdom), the Series Editor of *Methods in Molecular Biology*, for giving me the opportunity to select methods and protocols that I consider to be very important for research in MS. Therefore this book has a personal touch. The book covers molecular biology, cellular biology, biomarkers, imaging, and neuropathological methods and techniques. Moreover, special emphasis is put on disease models. The authors for this book were selected based on themes and research records.

Maja Jagodic and Pernilla Strindh (Stockholm, Sweden) give a state-of-the-art overview describing methods to clone genes in experimental populations that are relevant for MS. Holger Reichardt and Henrike J. Fischer (Göttingen, Germany) describe the generation of transgenic rats using lentiviral vectors. Such rats are potentially very potent tools to define relevant disease pathways and treatments for MS. Nicolas Fissolo, Xavier Montalban, and Manuel Comabella (Barcelona, Spain) educate in the generation of DNA vaccines. Animal modelling is a very powerful tool for research in MS. Experimental autoimmune encephalomyelitis (EAE) can be induced in different animal species and strains. E. Rachel L. Terry, Igal Ifergan, and Stephen Miller (Chicago, USA) describe ways to induce EAE in mice, myself in rats (Regensburg, Germany), and S. Anwar Jagessar, Karin Dijkman, Jordan Dunham, Bert t'Hart, and Yolanda S. Kap (Rijswijk, the Netherlands) in marmosets. Each species and strain has certain advantages and disadvantages in the modeling of MS. None of the models can imitate all aspects of MS, but more or less for each aspect of MS a specific model can be selected. Neuropathological techniques to investigate the CNS in EAE are described by Karin Steinbach and Doron Merkler (Geneva, Switzerland). Detailed neuropathological protocols are provided by Jan Bauer and Hans Lassmann (Vienna, Austria) to investigate lesions of patients with MS. Christian Schläger, Tanja Litke, Alexander Flügel, and Fracesca Odoard (Göttingen, Germany) present state-of-the-art two-photon laser scanning microscopy in rodents. Axel Petzold (Amsterdam, the Netherlands) educates on how to use optical coherence tomography in assessment of neurodegeneration in patients with MS. Purification of mononuclear cells from blood is described by Christine Riedhammer, Dagmar Halbritter, and myself (Regensburg, Germany). This method is important regarding standardization of sample collection and work up from patients with MS, since, in many centers around the world, the purification of mononuclear cells is the initial step in MS research. Niannian Ji and Thomas G. Forsthuber (San Antonio, USA) present the powerful Elispot methodology. Ilgiz A. Mufazalov and Ari Waisman (Mainz, Germany) describe ways to isolate cells that have infiltrated the CNS, and Darryl G. Turner, Melanie D. Leech, Richard A. O'Connor, and Stephen M. Anderton (Edinburgh, United Kingdom) explore how to analyze immune cell function from the CNS in EAE. There is a strong need to define novel disease biomarkers in MS. Marguerite Limberg, Giulio Disanto Christian Barro, and Jens Kuhle (Basel, Switzerland) present their method to quantify neurofilament light chains from the blood of MS patients. Melanie Spadaro and Edgar Meinl (Munich, Germany) demonstrate how to detect antibodies against myelin oligodendrocyte glycoprotein (MOG) in MS and related disorders. This is a very important

contribution that should help to standardize methodologies that are used in different laboratories around the world to detect anti-MOG-specific antibodies in MS. Maren Lindner and Christopher Linington (Glasgow, United Kingdom) present ways to identify demyelinating and axopathic autoantibodies in MS.

I am confident that the selected protocols and methods will empower the reader to perform novel research regarding pathophysiology and treatment for MS. There is still much effort to be done to elucidate basic disease mechanisms, to clone disease-relevant genes, to define novel biomarkers, and to discover novel and improved therapeutic and curative treatments. Finally, I want to thank David Casey (New York, USA), the Editor from Springer, for his continuous support in the production process of this book.

Regensburg, Germany *Robert Weissert*

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Part I

Molecular Biology Techniques

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Positional Gene Cloning in Experimental Populations

Maja Jagodic and Pernilla Stridh

Abstract

Positional cloning is a technique that identifies a trait-associated gene based on its location in the genome and involves methods such as linkage analysis, association mapping, and bioinformatics. This approach can be used for gene identification even when little is known about the molecular basis of the trait. Vast majority of traits are regulated by multiple genomic loci called quantitative trait loci (QTL). We describe experimental populations and designs that can be used for positional cloning, including backcrosses, intercrosses, and heterogeneous stocks, and advantages and disadvantages of different approaches. Once the phenotype and genotype of each individual in an experimental population have been determined, QTL identification can be accomplished. We describe the statistical tools used to identify the existence, location, and significance of QTLs. These different methods have advantages and disadvantages to consider when selecting the appropriate model to be used, which is briefly discussed.

Although the objective of QTL mapping is to identify genomic regions associated with a trait, the ultimate goal is to identify the gene and the genetic variation (which is often quantitative trait nucleotide, QTN) or haplotype that is responsible for the phenotype. By discovering the function of causative variants or haplotypes we can understand the molecular changes that lead to the phenotype. We briefly describe how the genomic sequences can be exploited to identify QTNs and how these can be validated in congenic strains and functionally tested to understand their influence on phenotype expression.

Keywords: Positional cloning, Gene identification, Linkage analysis, Association mapping, Quantitative trait loci (QTL), Quantitative trait nucleotide (QTN), Intercross, Backcross, Advanced intercross line, Heterogeneous stock, Inbred and congenic strains

1 Introduction

1.1 What Is Positional Cloning?

Positional cloning is a technique that identifies a trait-associated gene based on its location in the genome and involves methods such as linkage analysis, association mapping, and bioinformatics. In reality most traits are regulated by multiple loci called quantitative trait loci (QTL). The objective of QTL mapping is to identify genomic regions associated with a trait. In the absence of a simple relationship between a QTL and a trait, QTLs are mapped by linked genetic markers that segregate in a Mendelian fashion and can be unambiguously determined. If a QTL is adjacent to a genetic marker, the phenotypic values for the trait will differ between genotypes at the specific marker. The difference in phenotypic value between genotype groups will be larger the closer together

the QTL and marker are, and it will reach a maximum when the marker exactly coincides with the gene. Given a genetically segregating population that originates from strains that are variable for the phenotype, the whole genome can be systematically tested for the presence of a QTL through evenly spread informative markers (i.e., polymorphic between the strains used to create the mapping population). The segregation of markers with the QTL and association with the trait are then statistically modeled to provide evidence for the presence of a QTL. This approach can be used even when little is known about the molecular basis of the trait.

1.2 Experimental Populations

Localization of QTLs is possible in a mapping population, which allows association between a genetic marker and the trait. In practice it most often starts with a selection of suitable inbred strains. The difference in the phenotype between inbred strains (each genetically identical and homozygous) kept in the same environment is solely a reflection of differences in variants of their genes. The main factor determining the precision of the QTL position is the recombination rate. Recombination events occur during the pachytene stage of meiosis through the introduction of breaks and exchange of material between chromatids. A variety of different breeding strategies leads to the remixing of parental genomes and creation of new genotype combinations. The use of crosses between inbred strains provides high power to detect linkage due to one round of recombination and all individuals are informative. Intercross (F2) and backcross (BC) populations are most frequently used in QTL mapping. An F2 cross generated by interbreeding F1 hybrids (heterozygous throughout the genome) is more informative and allows identification of both dominant and recessive QTLs in the same cross compared to a BC. The latter, generated by interbreeding F1 hybrids with one of the parental inbred strains, is more suitable than an F2 for mapping QTLs of major effect (1) and parent-of-origin effects (2). A few recombination events in both F2 and BC offspring allow a whole genome scan with a limited number of genetic markers. To achieve higher resolution an efficient approach is to increase the actual number of recombination events without a significant increase in population size. This can be achieved by creating an advanced intercross line (AIL). An AIL is created by randomly intercrossing two inbred strains for several generations, avoiding sister-brother mating (3). This scheme generates genetically unique offspring with a dense mixture of founder chromosomal fragments. Intercrossing causes accumulation of recombination events leading to more precise QTL mapping. The most important requirement is a minimum of 100 individuals in the breeding population in each generation to reduce allele fixation by genetic drift. Even with this size the exponential decrease in confidence interval will continue until approximately the tenth generation, after which it will display a rather stable course. An additional

requirement is a proportionally higher marker density necessary to achieve an equivalent power to detect QTL as in an F2 cross. To achieve a higher magnitude of resolution an efficient approach is to increase both the number of founding genomes and the number of recombination events, although this requires an increase in population size. A heterogeneous stock (HS) is a population that harbors recombinants derived from inbred strains that have accumulated over many generations of outbreeding to create a genetic mosaic. An HS is established from eight inbred strains (4, 5). By using a standard pseudo-random outbreeding schedule, inbreeding is minimized and recombination density is maximized to reduce the size of inherited haplotypes (6). This approach also reduces allele fixation by genetic drift, which enables breeding the population beyond 50 generations to accumulate recombination events and mix the founding genomes. Therefore, an HS can provide mapping resolution that is exponentially higher than an AIL, allowing fine mapping of QTLs to intervals smaller than a cM (7). Theoretically, it is possible to perform a genome-wide association study of complex traits in the HS, to identify and fine-map QTLs in the same population. Thus, this approach combines the gene identification step usually performed in BC/F2 populations with the fine-mapping step usually done in interval-specific recombinant congenic strains and advanced populations (8). The size of the mapping population required for QTL detection depends on the phenotype variance in the population and the number of QTLs and their effects, which is difficult to predict. In general, a larger population gives a greater chance of detecting several QTLs, even those displaying small effects. Overall, as genetic complexity increases to match complex phenotypes, experimental control and statistical simplicity decrease. The populations that offer the most control and statistical confidence are also the most genetically homogeneous populations (inbred and congenic strains), and therefore the most artificial models. On the other hand, populations that offer the highest probability of capturing the complexity involved in multifactorial traits are heterogeneous populations (HS) and are therefore more sensitive to the influence of family structure and confounders. To cope with this, rigorous statistical analyses using stringent criteria are necessary. This may partly explain why second generation crosses, which are in the middle of the complex genetics-statistical control continuum, are so widely used in genetic research.

1.3 Genetic Markers

A genetic marker is any polymorphic Mendelian character that could be used to follow a specific genomic location (i.e., to establish the strain of origin of inherited allele). The development of DNA markers in the early 1980s facilitated the era of whole-genome screening. PCR amplification of microsatellite markers revolutionized genomic screening. Microsatellites are simple di-, tri-, or

tetranucleotide repeats. They are highly heterozygous, easy to score by standardized PCR, and relatively frequent (every 10–20 kb). The newest generation of markers, i.e., single nucleotide polymorphisms (SNP), despite their bi-allelic form are introduced due to the ultrahigh-throughput genotyping capabilities and high density (approximately >3 million SNPs between any two given strains). The development of dense genetic maps in different organisms followed the discovery of new markers and suitable detection methods. The spacing of markers required to achieve power enough to detect QTLs depends on the QTL effect and the type and size of the mapping population. Marker spacing of 10–25 cM is often accepted in F2 and BC populations. To achieve the same power in an AIL as in an F2 or BC, marker spacing of 1–5 cM is required. Microsatellites are still the genetic markers of choice in the rat. They are numerous and can provide <1 cM coverage in certain strain combinations. Detection is based on well-standardized PCR and linkage and physical maps are available. Despite more than 30 rat strains being sequenced and rapid SNP discovery, development of inexpensive SNP genotyping methods in the rat is still in its infancy.

1.4 QTL Identification

Once the phenotype and genotype of each individual in a mapping population have been determined, statistical tools to identify the existence, location, and significance of QTLs are applied. There is a distinction between methods testing single markers and intervals as well as methods assuming the existence of one or multiple QTLs. These different methods have advantages and disadvantages to consider when selecting the appropriate model to be used, which will be briefly discussed. Model selection may never be perfect, but choosing an incorrect model can be detrimental. The simplest method performs analysis at a single marker (9). It compares phenotypic expression between groups of experimental animals stratified according to their genotypes at a given marker using a simple *t*-test, analysis of variance, or any nonparametric test. If the genotype groups differ in phenotype there is an indication that the marker is linked to a QTL. If they are the same the marker is not linked to a QTL. The main advantage of this method is simplicity and no need for a genetic map (i.e., the order of genetic markers and distance between them) and special software. A particular strength of the method is that it can be easily extended to account for multiple covariates and QTLs. The main disadvantage is that the QTL location is imprecise because it depends on the marker location and estimated effects, assessed on marker genotypes, are always smaller than in reality. Furthermore, the method suffers from decreased power since all individuals with missing genotypes have to be excluded from the analysis. This has a most prominent influence when genotyping is performed on a coarse scale. We still use single-marker tests to confirm our findings and rule out the

possibility of false positives due to coarse genotyping, incorrect marker order, or violation of trait normality. Interval mapping (IM), developed by Lander and Botstein (10), is the most popular method for QTL analysis. Testing for a putative QTL is performed by "walking" along the genetic map. It is possible to calculate the probability of an individual's genotype at a putative location, depending on the genotypes of the nearest flanking markers. Thus additional information is gained from the relationship between markers. The strength of evidence is measured by LOD scores ("logarithm of odds" favoring linkage). A LOD score measures the strength of evidence for the likelihood of QTL presence given the data compared to the likelihood of no QTL at the position. Maximum likelihood (ML) is obtained where parameters are estimated to give the highest probability for the observed data (maximal value). ML uses a reiterative process of associating a trait to a genomic location based on probability and then reevaluating linkage with newly created information until a QTL is detected. IM has several advantages: (1) QTL can be more precisely localized, (2) estimation of the QTL effect is greatly improved, and (3) missing genotypes and errors are accounted for. Statistical stringency is used to correct for the multiple tests performed in order to decrease the risk of false positive QTLs. The relative disadvantage is heavy computations that require specialized software. Haley and Knott have developed a multiple regression method that remarkably well approximates IM (11). Moreover, it could easily be extended to multiple QTLs and covariate analyses while being much less computationally intense. Both single-marker and IM analyses assume a model with a single QTL. Mapping multiple QTLs simultaneously has several important advantages: (1) increase in power, (2) separation of linked QTLs, and (3) mapping epistatic interactions. Epistatic interactions are largely unpredictable and multiple QTL models might help in mapping regions that would never come up in single-QTL analysis. The *p*-value reflects the probability of obtaining an equal or larger LOD score than that observed in the case where there are no QTLs in the population. Very small *p*-values indicate that the QTL really exists. Corrections must be made for testing a hypothesis on a number of locations. Lander and Kruglyak performed extensive simulations and derived rather stringent but robust criteria for suggestive and significant linkage of 2.8 and 4.3 LOD scores, respectively in an F2 intercross with 2° of freedom (12). Another very attractive approach introduced by Churchill and Doerge is the permutation test (13). The use of permutation provides thresholds for significance specific for the performed experiment. It is based on the assignment of mismatched phenotypes and genotypes, recording the acquired maximum LOD scores and estimating how often a certain LOD score occurs in the population. The conventional permutation method cannot be used to set the significance thresholds in the AIL, due to the different

family structure compared to F2 generations for which it is developed. To set significance thresholds in an AIL, the within family variance (inheritance of phenotype with the causing genotype, i.e., linkage) is removed to determine LOD scores for between-family variance (representing random effects, i.e., no linkage) (14). Further confirmation of true QTL existence comes from reproducibility in independent experiments. Among many available software, we will focus on R/qtl (15). The R/qtl software introduced a number of options for QTL mapping and it allows both single- and two-QTL mapping using ML, Haley-Knott regression, and multiple imputation methods. Furthermore, R/qtl provides other models such as binary, nonparametric, and two-part models. The nonparametric model is based on Kruskal–Wallis statistics and is well suited for phenotypes not fulfilling normality criteria. In the case of a spike in the phenotype distribution the two-part model, which is a combination of binary and nonparametric models, provides maximum extraction of information. Moreover, models assuming the existence of more QTLs and/or interactions between them can be tested with multiple imputation. An additional feature is the permutation test for setting up the threshold for significant linkage. At the moment, R/qtl provides more methods and models than any other software. The association studies in HS involve a more specialized statistical approach to correct for the population structure created by the different degrees of genetic relatedness between individuals (16). The multitude of methods developed for genome-wide analyses in classical intercrosses is not applicable to this population (17–19). Therefore, novel analytical methods and statistical packages were developed specifically for the population to detect haplotype association (7, 8), Resample Model Averaging and Mixed Models. For normally distributed phenotypes, the preferred method is the Mixed Models approach (20), in which the population structure and genetic relatedness is corrected to reduce false positives in association mapping. Pairwise genetic relatedness is incorporated into the statistical model to account for that two genetically similar individuals are more likely to have correlated phenotypes than two genetically dissimilar individuals. The haplotype reconstruction phase of analysis is carried out using the R/HAPPY software. The QTLs are then fitted using R/EMMAX. The false discovery rates of identified QTLs are calculated to determine significance. For non-normally distributed phenotypes, the Resample Model Averaging method is used. Parental haplotypes are reconstructed, using a hidden Markov-chain approach, to predict probabilities of inheritance from each of the eight progenitor strains for each SNP. A multiple QTL model is then fitted using a model averaging method to obtain a posterior probability that a QTL will be included in the model (7). This is accomplished by repeatedly resampling the data and in each resample test which set of markers best explains the variation in the phenotype. Hence, the

association between phenotype and genotype at any one locus is corrected by the pattern of associations over the rest of the genome. The haplotype reconstruction phase of analysis is carried out using the R/HAPPY software. The QTLs are then fitted using R/Bagphenotype.

1.5 Validation of a QTL and Functional Testing

The size of a QTL identified in F2 or backcross usually ranges from 10 to 30 cM, containing several hundreds of genes that cannot all be evaluated. Above all, QTL mapping represents a statistical approximation of both the existence of QTL and it's genomic location and requires further confirmation before enormous funding and effort are put into gene identification. One approach that can solve both problems is mapping using congenic strains. A congenic strain is an inbred strain in which one part of the genome has been transferred from one strain (donor) to the other (recipient) by repetitive backcrossing to the recipient strain and by selection of animals having the region of interest from the donor strain. Ten generations of backcrossing are used as a standard to remove most of the contaminating donor genome outside the region of interest. The obvious weakness is time (3–4 years in rats) necessary to develop a congenic strain. The "Speed congenic" approach represents marker-assisted breeding in which a selection of animals that contain the region of interest is additionally selected for containing the lowest percentage of contaminating donor genome (21). A simulation analysis demonstrated that 16–20 males screened every 25 cM can create a congenic strain in five generations with an equivalent level of contamination as compared to the conventional ten generation backcrossing strategy. The use of higher marker density and number of males does not influence the rate of removal of the contaminating donor genome. The phenotypic difference between the parental and congenic strains, which only differ in the transferred region, demonstrates the influence of the QTL as identified in linkage analysis. Furthermore, by backcrossing the congenic with the recipient strain and selecting recombinants within the region, correlation of gradually smaller regions with a trait can narrow down the QTL allowing final gene identification (22). Correlation of the region and/or gene with a subphenotype may give insights into mechanisms of gene regulation and trait development. Extensive analysis could be performed repeatedly in genetically identical material that is easy to reproduce.

1.6 From QTL to QTN Identification

Although the objective of QTL mapping is to identify genomic regions associated with a trait, the ultimate goal is to identify the gene and the SNP(s) (quantitative trait nucleotide, QTN) or haplotype that is responsible for the trait. By discovering the function of causative SNPs or haplotypes we can understand the molecular changes that lead to the phenotype. The genomic sequences can be exploited to identify QTNs. The limiting factor is genetic

resolution, and this type of analysis requires either more a priori information or several generations of intercrossing and a large cohort to test in order to generate sufficient resolution. In the simplest of cases, a candidate gene is already established and the task is to identify the responsible nucleotide. Based on this information, the sequence differences between inbred strains or congenic and parental strains can elucidate the molecular change for the candidate gene to generate a hypothesis for testing. In most cases, more complex cross populations are used to identify QTN, much in the same manner as described above for QTL identification. One strategy that can be used to fine-map a previously identified region of interest is a Partial Advanced Intercross (PAI). The genomic region is first captured in a congenic strain that is then used to breed an intercross that only varies in the congenic region. The population is phenotyped and the SNPs in the variable region are typed to identify QTN by linkage analysis. The approaches described above are used to study a particular gene or genetic region, often based on a previous finding. However, QTN can also be identified on a genome-wide scale. To identify causative variants for genes and pathways associated with a trait, sequence-based and genetic mapping approaches can be combined. Genetic mapping on fully sequenced individuals is transforming our understanding of the relationship between molecular variation and trait. The HS has three characteristics that make it particularly well suited for this type of analysis: (1) the genetic resolution enables direct identification of risk genes; (2) the complete genomic sequence of genotyped HS animals can be imputed with high accuracy from the progenitor genomes, and (3) the population's well-defined haplotype space can be exploited to determine whether genetic association is caused by single variants or by haplotypes (23). This type of analysis requires knowledge about the segregating SNPs in the population. The HS permits a test, called merge analysis, of whether an SNP is responsible for the phenotype, or the combination of variants from a single progenitor, i.e., a haplotype, is causal. The test is possible because the haplotypes segregating in an HS are known (the stock is derived from the eight sequenced genomes). Any imputed variant that exceeds the maximum haplotype logP is termed a candidate causative SNP. The near complete sequence allows identification of multiple QTNs at a locus in addition to haplotypic effects. Defining a catalogue of QTNs enables the study of their effects on protein, mRNA, and gene regulation levels to understand their molecular genetic mechanisms. Manipulation of genes (using various gene targeting approaches) affected by the QTNs enables the study of their effects on the trait.

2 Materials

1. Experimental population. Inbred strains that will be used to generate experimental mapping population should be selected based on the phenotypic and genotypic differences. Inbred strains are families of animals where all members are genetically identical, or very close to identical. This is achieved by breeding brother and sister pairs for a minimum of 20 generations, which should achieve more than 99 % identical genome (24). Extensive information about phenotypic features of rat strains and genetic diversity between them can be found at the Rat Genome Database, RGD (http://rgd.mcw.edu/).
2. DNA extraction. Materials for DNA extraction will depend on the selected method. Any method or kit that isolates genomic DNA and avoids excessive fragmentation of DNA is suitable (e.g., conventional DNA precipitation with isopropanol or phenol–chloroform extraction).
3. Phenotyping. Materials for phenotyping depend on the equipment and assays needed for measuring the phenotypic expression of the trait that will be studied.
4. Genotyping. For the genotyping of microsatellite markers, consumables and equipment that are specific to the preferred PCR reaction or kit and the available capillary sequencer are required. We recommend multiplexing microsatellite markers, which can be done with for example Type-it microsatellite PCR kit (Qiagen). Although there are many machines and software available for fragment analysis, we have good experience with ABI 3730 DNA analyzer and GeneMapper® Software (Applied Biosystems). The genotyping of SNPs is often based on array methodology and thus performed commercially or in specialized laboratories.
5. Software. We recommend R/qtl software (15). Instructions to install R and the add-on package qtl and perform analysis can be found at http://www.rqtl.org/.

3 Methods

3.1 Strain Selection

Select inbred strains with the largest possible difference in the phenotype of interest and that are genetically diverse. Prior to the breeding, animals should be kept in the new facility for a minimum of 1 week to adjust. Breeding animals should be of an appropriate age, e.g., around 2 months old (rats or mice).

3.2 Experimental Population

Breed a desired experimental population. Backcross or intercross populations are suitable for determination of the number of QTLs and their location in the genome. All genotypes are obtained from the same set of parents, which means that the family structure of the population is known and allows straight and accurate linkage mapping without adjustments to the data, and statistical methods can reliably calculate significance thresholds (25). The mapping resolution in an intercross can be slightly better compared to a BC, because both chromosomes in each pair are allowed to recombine. On the other hand, a backcross is better suited for parent-of-origin analysis. However, QTLs detected in backcross and intercross populations comprise large intervals and often contain hundreds of genes. Higher mapping resolution is required for candidate gene selection and can be achieved in advanced intercross lines and especially in heterogeneous stocks. For example, using the 10th generation AIL (G10) provides an approximate 3.5- to 5-fold increase in resolution compared to an F2 intercross. An HS can provide mapping resolution that is exponentially higher than an AIL, and can thereby combine the gene identification step usually performed in BC/F2 populations with the fine-mapping step usually done in complex populations and congenic strains (8). A disadvantage in using a more complex population is that it does not meet the assumptions of established statistical methods. The statistical approach used to map linkage and establish significance thresholds and confidence intervals must be adjusted for the underlying population stratification.

3.2.1 Backcross (Fig. 1)

N2 backcross (BC) populations are created by backcrossing F1 hybrids to one of the parental inbred strains (26). By using an F1 hybrid as one parent and an inbred strain as the other, it is possible to determine which parent heterozygous alleles were inherited from (27). Additionally, by using F1 hybrid mothers in half of the population and F1 hybrid fathers in the other half (a reciprocal cross), a population is created in which the parental origin of trait-predisposing alleles can be established to determine parent-of-origin effects on inheritance (maternal, paternal, or shared) (2). Furthermore, by creating one reciprocal cross with the susceptible strain and another with the resistant strain, all three possible genotypes are obtained to enable most allelic effects to occur.

Example: To create the F1 generation, establish four breeding pairs. For reciprocal breeding, establish two pairs with B female founders ($F1_B$) and two pairs with W female founders ($F1_W$). The reciprocal N2 generation is created from B ($n \geq 4$) and W ($n \geq 4$) females bred to F1 males and F1 females bred to B ($n \geq 4$) and W ($n \geq 4$) males. If reciprocal founders are used, a minimum of 16 pairs with $F1_B$ and 16 pairs with $F1_W$ are set up. Several litters from each pair can be used for experimentation.

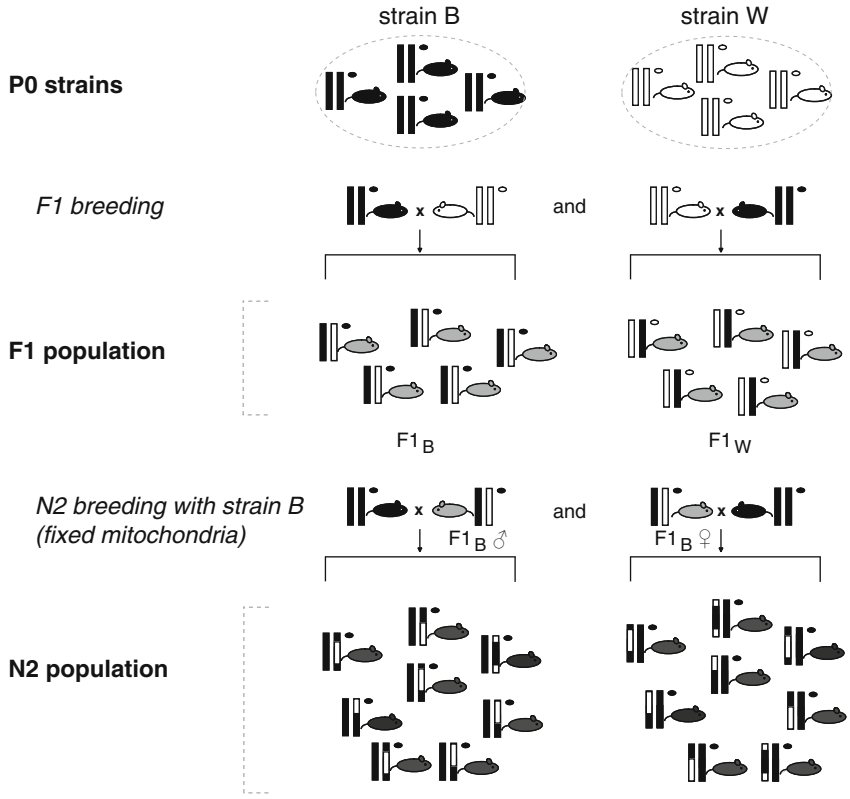

Fig. 1 Schematic illustration of the breeding setup used to create a reciprocal backcross population (BC). One pair of autosomes is represented by *vertical lines* and mitochondria are represented by *circles*. A BC population is useful for mapping QTLs, while also mapping parent-of-origin effects (mitochondria, sex chromosomes, and QTLs that depend on parental origin). Different breeding setups will allow these factors to vary (mitochondria, sex chromosomes, and maternal/paternal genotype origin), depending on the study aim. In this example, we are backcrossing F1 hybrids to strain B (allowing parental origin of B and W to vary) while keeping mitochondria fixed to B

3.2.2 Intercross (Fig. 2)

Alternatively, all three genotypes can be directly achieved in a population by mating two F1 hybrid parents. This is called an intercross and the most commonly used generation is the second (F2) (28).

Example: Create the F1 generation by the reciprocal breeding described for BC (under Sect. 3.2.1). The F2 generation is created from eight pairs with B female founders ($F1_B$) and W male founders ($F1_W$) and eight pairs with W female founders ($F1_W$) and B male founders ($F1_B$). Several litters from each pair can be used for experimentation.

3.2.3 Advanced Intercross Line (Fig. 2)

An AIL can provide a more precise QTL location and can reduce the interval it spans (3). The population is created much the same as F2 intercross populations, with the crucial difference that the breeding is continued in a pseudo-random fashion for several generations.

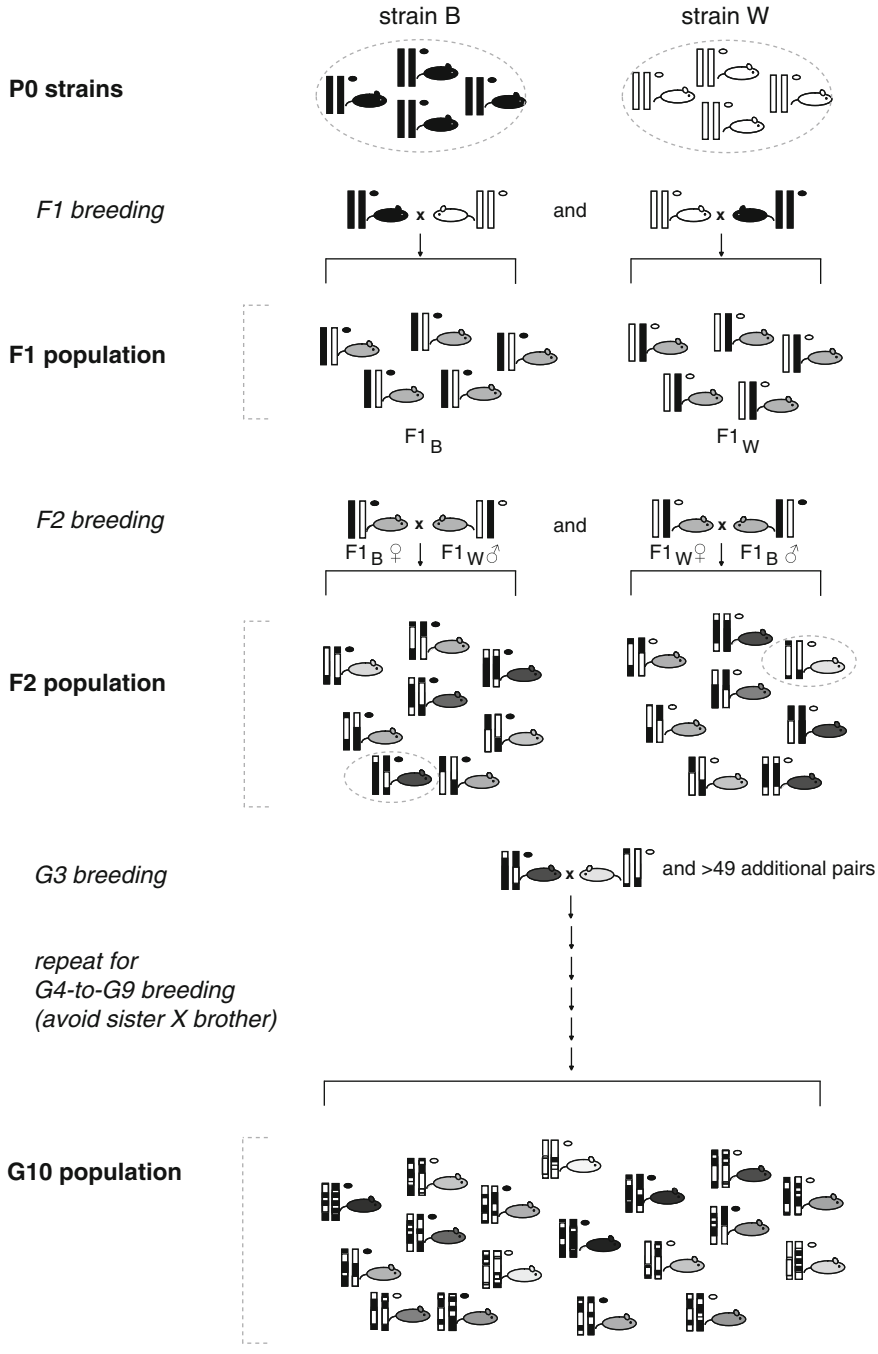

Fig. 2 Schematic illustration of breeding design for intercross (F2) and advanced intercross line (AIL) construction. One pair of autosomes is represented by *vertical lines* and mitochondria are represented by *circles*. An intercross captures all three possible genotypes in one population, which is useful for mapping dominant, additive, and recessive QTLs and QTL interactions. To create an F2 population, F1 hybrids are intercrossed. To create an AIL, intercrossing continues for additional generations, avoiding sister-brother mating

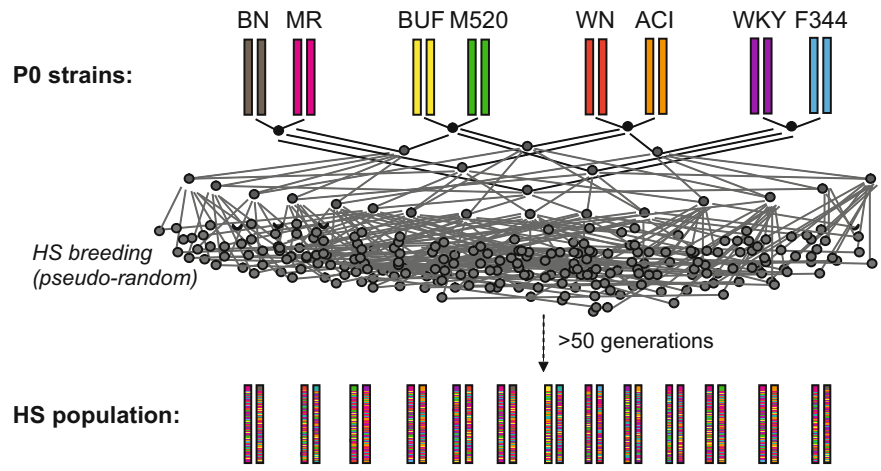

Fig. 3 Schematic illustration of heterogeneous stock (HS) construction. One pair of autosomes is represented by *vertical lines*. Eight inbred strains are intercrossed for more than 50 generations in a pseudo-random fashion to create genetic mosaics

Example: Create the F1 and F2 generations described for intercross (under Sect. 3.2.2). The G3 generation is created from breeding couples ($n \geq 50$) with both types of female founders (25 pairs each). Random breeding of 50 males and females, avoiding sister-brother mating ($n \geq 50$), creates all subsequent generations.

3.2.4 Heterogeneous Stock (Fig. 3)

The HS was established from eight inbred strains (4). The stock has been bred according to a standard pseudo-random outbreeding schedule more than 50 generations, using 40 breeding pairs for each generation. The breeding scheme is designed to minimize inbreeding and maximize recombination density to reduce the size of inherited haplotypes (6).

Example: There are HS colonies already established in both rat and mouse. The rat HS was established by Dr. Carl Hansen at the National Institutes of Health (NIH) in the 1980s from ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N, and WN/N (4). The MR, WN, and WKY strains trace their ancestry to the original Wistar stock, the ACI strain is a hybrid between the August and Copenhagen strains, the BN strain traces its ancestry to the Wistar Institute stock of wild rats, and the M520, F344, and BUF strains are of unknown origin. The European HS colony was established in 2004 by Dr. Alberto Fernandez Teruel at the Autonomous University of Barcelona obtained from the Northwestern University colony (Dr. Eva Redei). The mouse HS was established from A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, CBA/2J, DBA/2J, and LP/J (29). The AKR, C57BL, C3H, and BALB

strains were originally obtained from Charles Rivers Laboratories (Wilmington, MA) and A, CBA, DBA, and LP strains were obtained from Jackson Laboratories (Bar Harbor, ME). The stock was created by Dr. Robert Hitzemann in the 1980s and is currently maintained in his laboratory at the Oregon Health & Science University. The HS colonies are maintained using a circular breeding design, i.e., family one male is bred to family two female, family two male is bred to family three female, etc., to keep the genetic heterogeneity while reducing allele fixation. New HS populations can also be created by eight-way intercrossing inbred strains, but generating new stocks demands a lot of time and resources and we recommend using existing HS if possible.

3.3 Phenotyping

Measure your phenotype of interest in the entire experimental population. Measurements should be done as accurate as possible and all factors that can affect the phenotype should be appropriately recorded (e.g., sex, age, set, season, experimenter, reagents batch number, etc.). Collect relevant tissues at the end of experiment for potential follow-up studies.

3.4 Genotyping

Select genetic markers that cover the genome on an appropriate interval. Marker information (position and primer sequences) can be found at www.ensemble.org and www.rgd.mcw.edu (which also provides information about strain differences). To be informative, a marker must be polymorphic between the parental strains, i.e., have different numbers of repeats or different nucleotide (A, T, C, G). The effect of the QTLs being mapped, together with the type and size of the population used, dictates the appropriate interval for marker spacing to achieve power to detect the QTLs. In general, marker intervals of 10–25 cM are appropriate for intercross and BC populations and approximately 1–5 cM marker intervals are appropriate for comparable power in the AIL. The HS requires 100 times more markers than a cross from inbred strains to allow haplotype reconstruction (16). Extract genomic DNA from tissue biopsies, i.e., tail tips, ear clips, or any other tissue collected at the end of experiment (*see* **Note 1**). Genotype markers using your selected assay and protocol (*see* **Note 2**).

3.4.1 Microsatellites

Genotypes can be determined by PCR amplification of microsatellite markers. Microsatellites are highly heterozygous di-, tri-, or tetra-nucleotide repeats that differ between different inbred strains in the number of repeats and thus the size of the fragment amplified using primers that anneal to the unique DNA sequence flanking the repeat region. Fluorophore-conjugated primers are used and PCR products are size fractionated on capillary sequencer (*see* **Note 3**). Genotypes are analyzed using software; however, we recommend manual confirmation of genotypes for quality assurance.

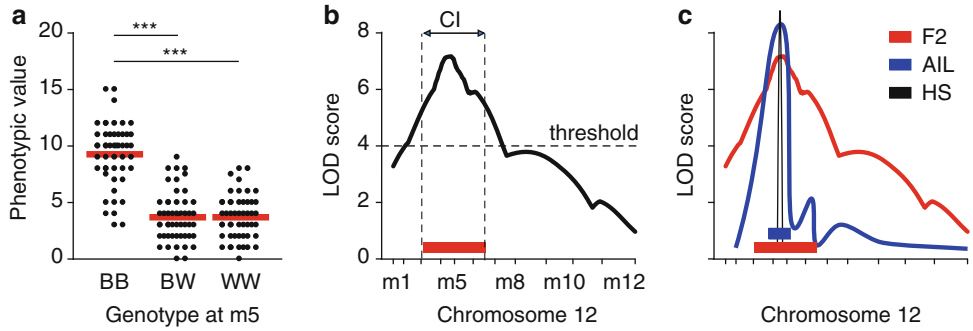

Fig. 4 Different approaches are used for QTL identification. (**a**) A single-marker test compares phenotype values between animals grouped according to their genotype (BB, BW, and WW). In this example, animals with genotype BB at marker 5 express higher phenotype than animals with BW or WW genotype, indicating that this marker is linked to a QTL. (**b**) Interval mapping scans for a putative QTL along the genetic map, thus adding information between markers. The genetic map for chromosome 12 is shown on the *x*-axis and the LOD scores, which measure the strength of evidence, are shown on the *y*-axis. The QTL is most likely to be located at marker 5 (highest LOD score), with the 95 % confidence interval between marker 3 and marker 6. (**c**) QTL location and confidence interval are more precisely estimated in populations with higher genetic resolution (higher recombination frequencies). An F2 intercross (*red*) identifies broad QTLs that contain many genes, while AIL (*blue*) and HS (*black*) maps narrower QTL intervals

3.4.2 SNPs

SNPs are bi-allelic base pair substitutions that occur with high density (~800 bp) that enable ultrahigh-throughput genotyping and development of dense genetic maps. Select SNP markers based on the strain sequences you are using and purchase/design SNP assays to use for PCR amplification. Allelic discrimination can be performed in the lab using fluorescence-based technology, i.e., TaqMan SNP genotyping assays (Applied Biosystems). More often, SNP genotyping is performed using a custom array (i.e., Affymetrix RATDIV array for rat HS) commercially or in specialized laboratories. Briefly, the array interrogates several hundred thousands of SNPs chosen based on sequence data for your strains of interest.

3.5 QTL Identification

Once the phenotype and genotype data are compiled for all individuals in the population, the likelihood of existence, location, and significance of QTLs is statistically determined by applying a model to the data. For a quick or preliminary test to scan your data for QTLs, we suggest using single-marker tests. This simple method is quick, requires no special software or need for a genetic map. *More comprehensive analysis (given under Sections 3.5.2, 3.5.3, and 3.6) might require additional training or statistical and bioinformatics assistance.*

3.5.1 Single-Marker Tests (Fig. 4a)

Let us consider an experiment in an F2 cross. Group animals into three groups according to their genotype (BB, BW, and WW) and compare phenotypes between the groups. Select the appropriate

test for your data. ANOVA can be used if phenotypic values show normal distribution, while nonparametric tests are better suited for phenotypic values that deviate from normal distribution. A significant difference between genotype groups indicate that the marker is linked to a QTL and warrants more in-depth analysis (described below). Repeat this for every marker to identify all potential QTLs. A threshold for significance has to be established with more detailed analysis that takes into account the population structure, number of markers, individuals, and QTLs. For quick inspection we would consider everything with $p < 0.01$ as potentially interesting (given further follow-up).

3.5.2 Interval Mapping (Fig. 4b)

This method often entails heavy computations that require specialized software. There is a variety of software packages that can be used, and we will base our description on R/qtl, which is freely available (15). IM requires a genetic map, i.e., chromosomes and locations of markers, either physical based on the genomic sequence (Mb) or linkage based on recombination fractions in the population (cM). LOD scores are then generated in a reiterative process of associating the phenotype to genomic locations along the map and then re-evaluating linkage considering the newly created information until a QTL is detected. QTL are more precisely localized by this method, and missing genotypes and errors are accounted for to preserve power while multiple test corrections decrease the risk of false positive QTLs. Select the appropriate interval between steps for the analysis. In general, a BC or F2 population rarely has dense enough recombinations to warrant smaller steps than 5 cM while an AIL has accumulated recombinations and therefore warrants tighter mapping, usually 1–2 cM (depending on the size of the population). A good rule-of-thumb is that at least 1 % of the population should have recombined between two tested positions, which equals 1 cM in distance. Select the model to be used for QTL analysis. Please see the instructions for your software package regarding the models included. In R/qtl, standard interval mapping can be performed using the em model, while the simplified Hailey-Knott regression gives a very good approximation of em for normally distributed data. Other regression models include nonparametric regression (non-normally distributed data), binary model (yes/no data), two-part model (a combination of binary and nonparametric models for data containing a spike in the phenotype distribution), and imputation where missing genotypes are imputed based on surrounding marker genotypes. Once you have analyzed your data, select the most appropriate method for setting significance thresholds and confidence intervals. For BC and F2 intercross, standard methods of permutation and bootstrapping can be used. Permutation provides significance thresholds that are specific for the study. Essentially, the genotypes and phenotypes are mismatched before QTL analysis

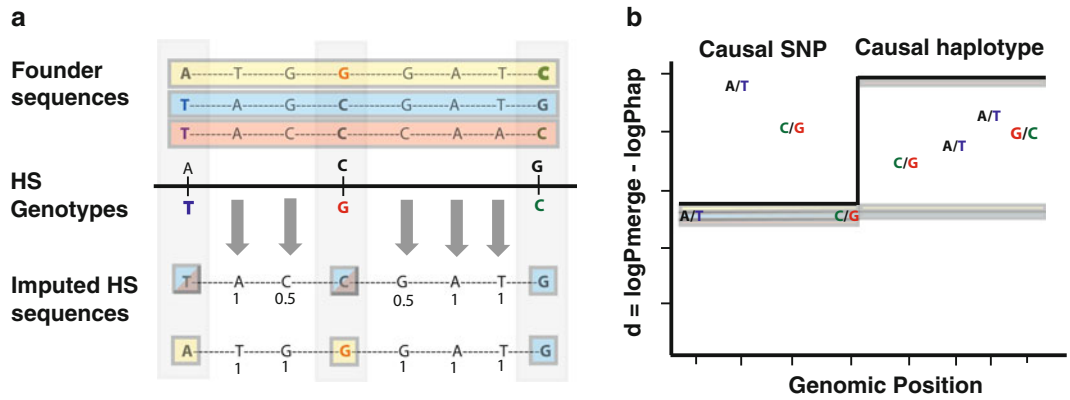

Fig. 5 The genomic sequence of each HS animal is imputed from the HS founder sequences, based on a selection of genotyped SNPs. (**a**) The genotypes in the experimental population are distinguished by reconstructing the parental haplotypes to predict the probabilities of inheritance from each of the eight progenitor strains for each SNP. The SNPs and haplotypes are then tested for association. (**b**) The sequence-based and genetic mapping approaches can be combined to test whether a single SNP or a haplotype (the combination of variants from a single progenitor) is responsible for the phenotype, the so-called merge analysis. Any SNP logP that exceeds the maximum haplotype logP is a candidate for causal functional variants (Causal SNP)

and the maximum LOD scores are recorded for a series of analyses (usually 1,000–2,000 but best 10,000) to estimate how often a certain LOD score occurs by chance in the population. The conventional significance threshold is 95 %, but other stringency can be used if desired. To account for the family structure in AIL, family residual values can be used to calculate significance thresholds. The within family variance (inheritance of phenotype with the causing genotype, i.e., linkage) is removed to determine LOD scores for between-family variance (representing random effects, i.e., no linkage) (14).

3.5.3 Association Mapping in HS (Fig. 5a)

Genome-wide SNP information can be used for genome-wide association studies (GWAS, or association mapping), given that the genetic resolution in the population supports such dense analysis. The GWAS in HS involve novel analytical methods and software packages developed specifically for this population (7, 8). To distinguish genotypes in the experimental population, parental haplotypes are reconstructed, using a hidden Markov-chain approach, to predict probabilities of inheritance from each of the eight progenitor strains for each SNP. Haplotypes are constructed for each rat across the genome using the multipoint haplotype reconstruction method HAPPY (http://www.well.ox.ac.uk/happy) implemented in R (16). The association studies in the HS involve a more specialized statistical approach, because of the population structure where individuals have different degrees of genetic relatedness (16). There are two strategies for dealing with relatedness: Mixed Models

in which the genotypic similarity between individuals is used to model their phenotypic correlation (20) and Resampling-based Model Inclusion Probability (RMIP) in which loci that replicate consistently across multiple QTL models fitted on subsamples of the mapping population are identified (30). In both strategies, QTLs are detected by haplotype association (16). Mixed models perform better on normally distributed data (23). The variance components that correct for the genotypic similarity between individuals (pedigree relationships) are estimated using the EMMA package for R (20). The test for association is then performed via a mixed model and expressed as the negative $\log_{10}$ of the p-value ($-$LogP), and covariates can easily be incorporated into the model. The significance threshold is calculated as the $-$LogP that corresponds to the false discovery rate (FDR) established by permutation for each phenotype, conventionally set to 5 % FDR but other levels may be used. Conversely, RMIP performs better for non-normally distributed phenotypes and binary data. In this approach, a "multiple QTL model" is fitted using a model averaging method to obtain a posterior probability that a QTL will be included in the model (7). This is accomplished by repeatedly resampling the data and in each resample test which set of markers best explains the variation in the phenotype. In short, the association between phenotype and genotype at any one locus is corrected by the pattern of associations over the rest of the genome. The QTLs are fitted using R/Bagphenotype. The significance threshold is calculated as the RMIP that corresponds to the false discovery rate (FDR) as described for Mixed Models.

3.6 QTN Identification in HS (if Applicable) (Fig. 5b)

The sequence-based and genetic mapping approaches can be combined to identify causative variants for genes and pathways associated with the phenotypes. If the founder strains are sequenced, the origin of each variant in the population is known. In the HS, this totals approximately 7.2 million SNPs. We can use this catalogue of segregating SNPs to identify genes and causative variants. The HS permits a test, called merge analysis, of whether a single SNP is responsible for the phenotype, or the combination of variants from a single progenitor, i.e., a haplotype, is causal (23). The test is possible because the haplotypes segregating in an HS are known (the stock is derived from the eight sequenced genomes). Any SNP that exceeds the maximum haplotype logP is a candidate for causal functional variant. The near complete sequence of the HS rats allows us to determine the presence of multiple causal variants at a locus in addition to haplotypic effects. The functional consequences of identified causal variants can then be modeled and experimentally tested based on position in the genome. For example, the consequences on protein structure of candidate variants lying within coding regions of genes can be predicted by modeling the sequence to known protein structures to elucidate altered

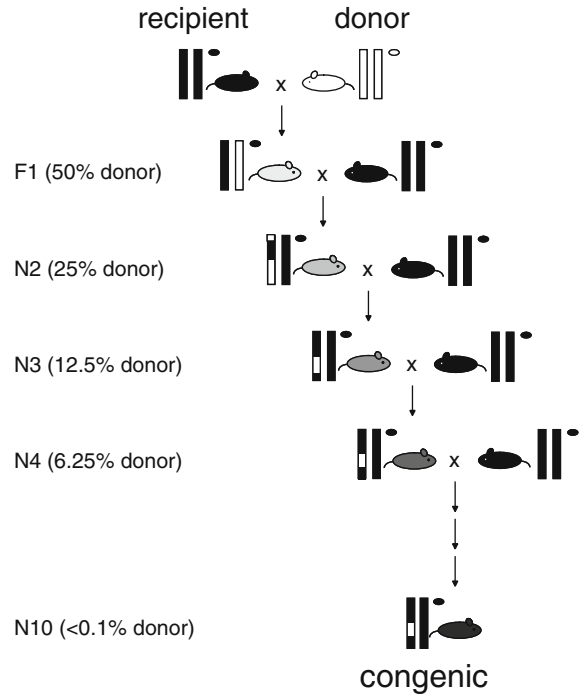

Fig. 6 Schematic illustration of congenic strain construction. One pair of autosomes is represented by *vertical lines* and mitochondria are represented by *circles*. Congenic strains are strains that carry isolated genes (positionally cloned or disrupted) from a donor strain (B or W) onto a genetic background of different susceptibility (recipient strain). They are backcrossed to the recipient strain for ten generations to ensure that the genomic background has minimum contamination with fragments of donor DNA. Congenic strains are used to validate the QTL and elucidate mechanisms underlying gene actions that contribute to the trait

binding affinities and physical interactions. For noncoding causative SNPs, regulatory sequence similarities and changes in transcription factor binding motifs can be modeled. The ultimate functional test entails capturing the sequence alternatives for causative haplotypes or SNPs in a congenic/transgenic/knock-in animal to test for functional consequence.

3.7 Validation Using Congenic Strains (Optional Step) (Fig. 6)

We highly recommend QTL validation before proceeding with often costly and tedious gene and QTN identification. Animal strains that carry isolated genes (positionally cloned or disrupted), i.e., congenic strains, offer a unique opportunity to validate the QTL and elucidate mechanisms underlying gene actions that contribute to the trait. They are similar to inbred strains, with the exception that the genomic region of interest has been transferred from a donor strain (B or W) onto a genetic background of different susceptibility (recipient strain) (*see* **Note 4**). A congenic strain

can be produced by intercrossing two strains to create F1 hybrids, and then backcrossing the F1 to either parental strain (B or W). The genetic recombinations will create unique animals and the aim is to select one that carries the region of interest from the donor strain. They are backcrossed to the recipient strain for ten generations to ensure that the genomic background has minimum contamination with fragments of donor DNA. Alternatively, marker-assisted selection can be used (speed congenic), in which the background genome is screened to select the animal with least contaminating donor genome together with the region of interest, to establish a homozygous congenic strain in 5–7 generations. For detailed protocol on how to create a speed congenic strain see Wakeland *et al* (21).

4 Notes

1. DNA extracted from ear clips, commonly used to mark animals at a young age, can be used to perform genotyping prior to phenotype measurements. This can significantly speed-up the experiment as genotyping can be completed before the end of the experiment.
2. Initially, it is possible to genotype only animals that display extreme phenotypic values and perform analysis. However, we recommend genotyping all individuals.
3. Different fluorophores allow for multiplexing of microsatellite markers during the PCR step. It is however important to carefully plan the multiplexing procedure. The quality of the fluorophore signal in the sequencer will determine the number of different fluorophores that can be used to label primers. Theoretically, markers of the same size can be multiplexed if their primers are labeled with different fluorophores. In our experience, sometimes a signal from one fluorophore can be detected (leakage) in the channel of another fluorophore. Thus, we recommend that markers of different sizes should be used. Also in theory markers that differ in approximately 20 bp can be labeled with the same fluorophore and multiplexed. This will work well if markers have only few bands after PCR amplification. In our experience the best results are obtained with three fluorophores and up to eight markers that differ as much as possible in their size.
4. Ideally congenic strains should be constructed on both parental backgrounds (B and W). Practically this is not always possible due to allelic effects, trait architecture (the number of QTLs that affect the trait), QTL strength, etc. Let us assume that we use an F2 cross between W (expressing high phenotype values) and B (expressing low phenotype values), to identify five QTLs.

QTL 1 shows a 10 % effect size and at this loci W genotype drives low phenotype expression. In this case, constructing a congenic strain that carries W genotype for QTL 1 (low phenotype expression, 10 %) on a genomic background of B strain (low phenotype expression) does not give optimal conditions to detect a phenotype difference. Another factor to consider is costs, and one should carefully select combinations for breeding that are most likely to generate useful tools, i.e., that have a robust phenotype difference between congenic and parental strain.

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Generation of Transgenic Rats Using Lentiviral Vectors

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Abstract

Transgenesis is a valuable tool with which to study different aspects of gene function in the context of the intact organism. During the last two decades a tremendous number of transgenic animals have been generated, and the continuous improvement of technology and the development of new systems have fostered their widespread application in biomedical research. Generally, transgenic animals are generated by introducing foreign DNA into fertilized oocytes, which can be achieved either by injecting recombinant DNA into the pronucleus or by transferring lentiviral particles into the perivitelline space. While mice remain the favored species in many laboratories, there are a number of applications where the use of rats is advantageous. One such research area is multiple sclerosis. Here, several experimental models are available that are closely mimicking the human disease, and it is possible to induce neuroinflammation by transferring pathogenic T cells which can then be studied by flow cytometry and 2-photon live imaging. Unlike for mice, the development of transgenic rats has encountered some hurdles in the past, e.g., due to a complicated reproductive biology and the frailty of the fertilized oocytes in vitro. In this chapter we provide a protocol describing how we manipulate single cell embryos in our lab in order to efficiently generate transgenic rats in a variety of different strains using lentiviral gene transfer.

Keywords: Transgenesis, Rat, Lentivirus, Oocyte injection, Perivitelline space

1 Introduction

In the last four decades, transgenesis has become a key technology to tackle biomedical questions since no other tool allows for studying the complexity of gene regulation and function in the context of the intact organism. Previously, the analysis of spontaneous mutations had been the only possibility to address gene function in vivo, but those were sometimes difficult to identify and not available for many genes of interest. Besides transgenesis, knock-out technology is another highly valuable method to manipulate genes in vivo. Nonetheless, until recently this technique was limited to mice as the derivation of pluripotent embryonic stem cells from the rat had failed for many years (1, 2). Hence, transgenesis was the only tool available in this species. To circumvent the unavailability of knock-out rats several alternative approaches have been employed over the years. These include ENU mutagenesis (3, 4), transposon-mediated insertional mutagenesis (5), and the generation of inducible knock-down rats by lentiviral delivery of shRNA

constructs (6). Nonetheless, even with the development of new technology aimed to delete genes in rats via embryonic stems cells (7, 8) or to introduce point mutations into their genome (9), e.g., with the help of Transcription Activator-Like Effector Nucleases (TALEN), traditional methods for the generation of transgenic rats are still highly valuable.

The first transgenic animal was created in 1976 through infection of mouse embryos with Moloney leukemia virus (10). Attempts to generate transgenic animals by transferring DNA fragments initially succeeded in 1981 (11–15). Later on, transgenesis was extended to other species including rabbits, sheep, and pigs (16) as well as rats (17). The use of lentiviral vectors for the generation of transgenic animals further advanced the application of this technique, especially to rats, as the efficacy of transgenesis was strongly improved and became technically more feasible. In the beginning, it was transgenic mice that were generated using lentiviral vectors (18), but in 2002 application of this technique succeeded in rats as well (19). In contrast to pronucleus injection, the use of lentiviral vectors allows to produce transgenic rats in basically every strain of rat including inbred ones such as Lewis (20, 21), Dark Agouti (22), and BioBreeding (23). Noteworthy, lentiviral vectors have some caveats. For instance they have a limited capacity of about 5–8 kb that can be cloned into the vectors (24, 25). Furthermore, lentiviral vectors integrate individually and therefore segregate in the offspring (26). Hence, lentiviral transgenesis is a valuable method but it might not always be recommended depending on the scientific question asked.

A tremendous number of transgenic mice have been reported in the past decades while the number of transgenic rat lines is much lower. This is amongst others due to the fact that the generation of transgenic rats has encountered some hurdles (27). The single cell rat embryo for example is surrounded by a robust *zona pellucida* making the introduction of the injection needle more difficult than in mouse embryos. In addition, reproduction biology is somewhat different from the mouse. Nonetheless, rats undoubtedly have their advantages as compared to mice in tackling specific scientific questions. For instance, they are larger which facilitates surgical techniques including organ transplantation. In multiple sclerosis (MS) research, there are a number of disease models that closely resemble human pathophysiology (28). Furthermore, there is a unique system where fluorescently labeled pathogenic T cells are adoptively transferred into recipient rats leading to neuroinflammation and clinical symptoms. Importantly, this model allows tracking and studying the transferred cells not only by flow cytometry but also by intravital 2-photon live imaging (29, 30). Hence, rat models of MS have allowed to obtain valuable insights into the pathogenesis of this highly prevalent autoimmune disease which otherwise would have not been possible.

Taken together, transgenic rats clearly have their justification despite the overwhelming use of genetically manipulated mice in biomedical research. In particular in MS research they have proven to be extremely valuable. To make this methodology more accessible to other laboratories we here report how we routinely generate transgenic rats by using lentiviral vectors.

2 Materials

2.1 Animals

1. Oocyte donors: Female rats at 11–12 weeks of age. There is a variety of strains that can be used including several inbred ones. We have successfully employed Lewis (20, 21), Dark Agouti (22), and BioBreeding (23) rats as oocyte donors. The males used for fertilization should be of the same strain as the females used for oocyte production.
2. Vasectomized males: We usually use Sprague Dawley or Wistar rats. These outbred strains are robust and show uncomplicated wound healing. Vasectomy is carried out in adult males at 12–14 weeks of age. The vasectomized males can produce pseudopregnancy in female rats until the age of approximately 12 months.
3. Foster mothers: We use outbred female rats, generally Sprague Dawley or Wistar, since they can easily handle large litters. Oocyte transfer is performed at the age of 12–15 weeks.

2.2 Hormones and Enzymes

1. GnRH analog ([des-Gly^{10}, D-Ala^{6}]-Luteinizing Hormone-Releasing Hormone ethylamide acetate salt hydrate; Sigma, Taufkirchen, Germany). 5 mg of GnRH analog is dissolved in 25 ml PBS and 0.2 ml of the resulting solution is used per i.p. injection.
2. hCG (human Chorionic Gonadotropin; Sigma). 5,000 IU is dissolved in 50 ml physiological sodium chloride solution; 0.3 ml is used per i.p. injection.
3. PMSG (Pregnant Mare's Serum Gonadotropin; Intervet, Unterschleißheim, Germany). 1,000 IU is dissolved in 10 ml physiological sodium chloride solution; 0.3 ml is used per i.p. injection.
4. Suprarenin® (Adrenaline; Sanofi-Aventis, Frankfurt, Germany). One drop of the 1 mg/ml solution is applied to the blood vessels of the *bursa* surrounding the ovary before cutting the ovary membrane.
5. Hyaluronidase (from Ovine Testes; Calbiochem, EMD Biosciences, Darmstadt, Germany). 10^5 IU is dissolved in 1 ml sterile H_2O and 100 µl aliquots are prepared. 100 µl is freshly

defrosted and diluted in 2 ml M2 medium (50 IU/ml) prior to usage.

6. All hormones and enzymes are stored at −20 °C.

2.3 Surgery

1. Narcotics: 10 % Ketamine; 2 % Xylazine.
2. Assorted surgical instruments: scissors, forceps, micro-serrefines, Dumont forceps, 2 mm blade spring-type micro-scissors.
3. Sterile, mounted swabs (Sugi; Kettenbach, Eschenburg, Germany).
4. Disinfection spray, razors, and—if necessary—a big scissor to cut the hair of the rats at the flanks.
5. Soldering iron for cauterization.
6. Catgut: supramid extra 3/0 USP for ligation of spermatic ducts (Hauptner Herberholz, Solingen, Germany); Marlin violet absorbable, 4/0 USP (Catgut, Markneunkirchen, Germany) for all sutures.
7. Spray plaster.

2.4 Embryo Preparation and Handling

We use heat-blunted fire-polished glass handling pipettes (Gündel BioMedical Instruments, Zöllnitz, Germany) with different diameters depending on each handling step of the oocytes.

2.4.1 Handling Pipettes

- 195–204 μm for initial transfer of the oocytes into the medium (type I).
- 155–164 μm for cleaning of the oocytes after hyaluronidase treatment (type II).
- 125–134 μm for transfer of oocytes onto the microscope slides (type III).
- 108–114 μm for retransfer of injected oocytes into the oviduct (type IV).

2.4.2 Additional Pipettes and Needles

- Lentivirus injection needles GC100TF-10, fire-polished, ID ~ 3 μm, L ~ 8 mm, SW ~ 42,5°
- Holding pipette standard; GC100T-15, fire-polished; ID = 20–25 μm, straight

2.4.3 Media and Solubles

- M2 and M16 medium (Sigma), sterile filtered prior to use.
- Mineral oil, embryo tested (Sigma).

2.4.4 Consumables

- In vitro fertilization dishes (BD Falcon, Heidelberg, Germany), tested for embryotoxicity.
- 6-well suspension culture plates; 10 ml Petri dishes; syringes and hollow needles.

- Sterile filters (filtropur S 0.45; Sarstedt, Germany).
- Microscope slides with a frame.
- Pipette microloader tips 0.5–20 μl (Eppendorf, Hamburg, Germany).

2.4.5 Microscopes and Other Devices

- Oocyte preparation: dissecting stereomicroscope (Leica MS5, equipped with 0.63–4× magnification objectives).
- Embryo injection: injection microscope (Zeiss Axiovert 35, equipped with 5×, 10×, and 20× magnification objectives); micromanipulator (PatchMan NP2, Eppendorf, Germany) and pressure control (CellTram vario, Eppendorf, Germany) for the holding pipette; microinjector for lentivirus injection needle (TransferMan NK2, Eppendorf, Germany) equipped with pressure supply (FemtoJet, Eppendorf, Germany).
- Surgery: stereomicroscope with 6–40× magnification (Leica M651).
- 37 °C incubator with 5 % CO_2.

3 Methods

3.1 Anesthesia

In general, anesthesia is conducted by i.p. injection of a combination of 10 % Ketamine and 2 % Xylazine at concentrations of 80 mg/kg and 5 mg/kg, respectively.

3.2 Vasectomy

3.2.1 Preliminaries

1. Narcotics: 10 % Ketamine; 2 % Xylazine.
2. Sterile surgery conditions and assorted surgical instruments: scissors, forceps, Dumont forceps.
3. Prepare sterile mounted swabs, disinfection spray, razors, and—if necessary—a big scissor to cut the abdominal hair.
4. Lay out a soldering iron for cauterization and catgut.

3.2.2 Surgery

1. Preferably use male outbred rats, e.g., Wistar, at the age of 10–12 weeks.
2. Shave the lower part of the abdomen and make a medial sagittal section (2.5 cm) at the caudal part of the abdomen. First incise the skin, followed by a section of the lower abdominal muscles along the *linea alba*. Locate the fat tissue at the lower right abdominal cavity. The fat should be easily pulled out and the right testes should move cranial out of the scrotum.
3. After location of the spermatic duct span it with small forceps and create two knots with catgut around the duct 1 cm apart from each other. In between those two knots the *vas deferens* is cauterized using a soldering iron. Finally, the testicle has to be carefully restored in the scrotum.

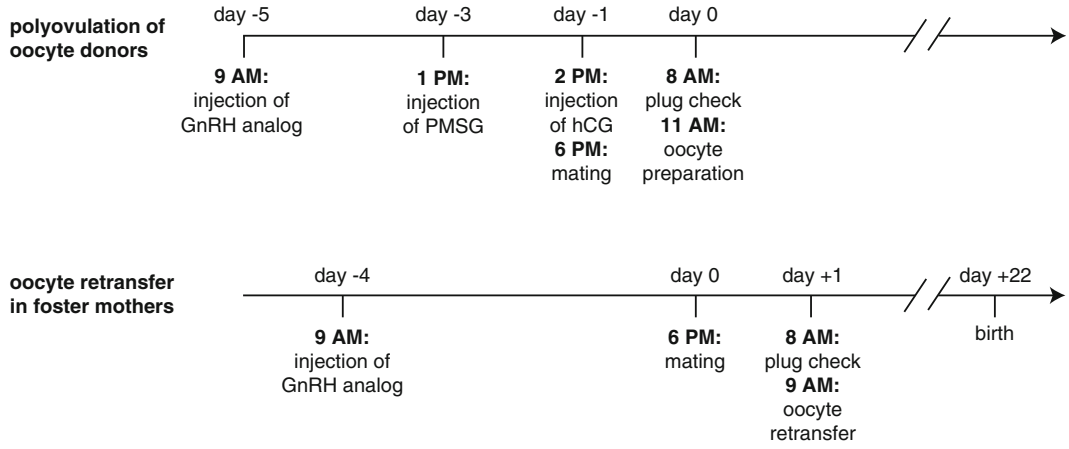

Fig. 1 Time schedule for oocyte production and retransfer

4. Repeat the same procedure with the second spermatic duct on the left side. Finally, the abdominal muscles and the skin are sutured separately and spray plaster is applied.
5. The animals should be given postoperative analgesia for 1 week. To verify that the vasectomy was successfully carried out, the males should be mated 5–6 weeks after surgery, followed by a pap test. A plug should be present but no spermatozoa visible under the microscope. We recommend to additionally check whether the females become pregnant (*see* **Note 1**). If both are not the case, it is safe to use the males for producing pseudopregnancy in foster mothers.

3.3 Polyovulation of Oocyte Donors

1. To induce polyovulation and oocyte maturation, female rats of your strain of choice are injected with a series of hormones three times (Fig. 1). We usually use eight embryo donors for one experiment.
   - First injection: 200 µl GnRH analog i.p. at 9 a.m. on day −5.
   - Second injection: 300 µl PMSG i.p. at 1 p.m. on day −3.
   - Third injection 300 µl hGC i.p. at 2 p.m. on day −1.
2. The rats are mated overnight on day −1 and the females are checked for a plug at 8 a.m. on the morning of day 0 (Fig. 1).
3. Oocyte isolation should start at 11 a.m. on day 0 (Fig. 1). Depending on the success of the hormone treatment up to 350 oocytes can be obtained from eight donors.
4. Strictly keeping to the indicated time schedule is crucial for successful oocyte production. Nonetheless, adjustments might be necessary depending on the dark-light cycle of your animal house (*see* **Note 2**).

3.4 Induction of Pseudopregnancy

1. The female recipient rats (foster mothers) are treated with a single dose of GnRH analog at 9 a.m. on day −4 (Fig. 1).
2. At the evening before oocyte transfer (day 0), the rats are mated with vasectomized males (Fig. 1).
3. On the next morning at 8 a.m. (day +1) the females are checked for the presence of a plug (Fig. 1). Only if a vaginal plug is present the rats can be used as foster mothers and a retransfer should be performed (*see* **Note 3**). In our hands two to three out of five mated females are plug-positive.

3.5 Isolation of Fertilized Oocytes

3.5.1 Preliminaries

1. Prepare sterile M2 and M16 medium by filtering.
2. Prepare the cover of a petri dish with droplets of M2 medium, four for each oocyte donor.
3. Prepare a 6-well plate: 3 ml M2 medium in well #1, a mixture of 1.5 ml M2 medium and 1.5 ml M16 medium in well #3, a mixture of 0.5 ml M2 and 2.5 ml M16 medium in well #4, and 3 ml M16 medium in well #5. Well #2 is reserved for the hyaluronidase treatment and contains a 100 µl aliquot of defrosted hyaluronidase diluted in 2 ml M2 medium (see below).
4. Prepare a 2 ml syringe with 2 ml M2 medium to be mixed with a vial of hyaluronidase immediately before treatment of the oocytes.
5. Lay out handling pipette type I and II (see Sect. 2.4.1) and a pipette holder with mouthpiece
6. Get the dissecting stereomicroscope ready with a fourfold magnification objective.

3.5.2 Isolation of the Oviducts

1. To isolate the oocytes, the donor females that were mated overnight are sacrificed and the abdominal cavity is opened. The two arms of the uterus are located at both sides.
2. Locate the oviduct and carefully separate it from both uterus and ovary. Note: It is advisable not to grab the oviduct directly with forceps but rather to hold the ovary. Also avoid pulling the oviduct too strongly because it might cause disruption. If the polyovulation was successful, the ovaries should appear red, not orange or yellow, and the swollen ampulla should be visible.
3. Place the oviduct in a fresh drop of M2 medium; repeat the aforementioned steps with the second oviduct.

3.5.3 Hyaluronidase Treatment and Oocyte Preparation

1. After transfer of the oviducts into the prepared medium droplets, the following steps should be conducted under the dissecting stereomicroscope with a 4× magnification objective.

2. The oocytes should be visible and surrounded by a cumulus mass in a swollen distinct part of the oviduct (ampulla). This part should be isolated using a Dumont forceps and a micro-spring-type scissor. Transfer the swollen part of the oviduct into a fresh droplet of medium and repeat the previous steps with the remaining oviducts.
3. The swollen parts should be incised making the oocytes to pour out, which then can be collected using type I glass pipettes (see above). All embryos are transferred in 2 ml M2 medium in well #1 of the prepared 6-well plate.
4. When all oocytes have been collected, they are jointly transferred into the M2 medium containing freshly defrosted hyaluronidase located in well #2 of the 6-well plate. After 1 min of incubation, the follicle cells of the *corona radiata* should dissociate from the oocytes. Naked cells can now be collected using a type II pipette. This process should be conducted as quickly as possible, since prolonged treatment with hyaluronidase causes the vitelline membrane to get flabby and hard to inject (*see* **Note 4**).
5. After hyaluronidase treatment the cells are transferred into well #3 of the 6-well plate containing 50 % M16 medium. This inhibits hyaluronidase activity; therefore it must not be used before this step. In the two subsequent wells of the 6-well plate containing increasing amounts of M16 medium (see above), the cells are cleaned from residual follicle cells by repeated pipetting and transfer steps.
6. Determine the number of oocytes and transfer them into pure M16 medium in an in vitro fertilization dish. Incubate for 3–4 h at 37 °C.

3.6 Injection of the Oocytes with Lentiviral Particles

1. Have the vial with the lentivirus ready on dry ice.
2. Lay out the lentivirus injection needle, the holding pipette, and microloader tips.

3.6.1 Preliminaries

3. Lay out handling pipette type III.
4. Prepare an in vitro fertilization dish with 1 ml M16 medium, mineral oil, a framed microscope slide, and the microscope with micromanipulators for the holding pipette and the injection needle.

3.6.2 Embryo Injection

1. The following steps must be conducted under biosafety level 2 conditions.
2. Prepare a framed microscope slide with a droplet of prewarmed M16 medium under sterile conditions. In this droplet the injection will be conducted.

3. To avoid contamination, the droplet should be covered with embryo-tested mineral oil. To this end, carefully top the inner lumen of the microscope frame using a 1 ml pipette.
4. Fill the injection needle using long microloader pipette tips, deaerate prior to injection.
5. Install the injection needle in the micromanipulator at the microscope, set the pressure 776 hPa, and ensure permanent flow of virus solution.
6. Install the holding needle in the other micromanipulator and fill it to two thirds with medium.
7. Load the embryos into the medium droplet on the microscope slide in batches of 30 using a type III handling pipette.
8. Pick up single oocytes with the holding pipette and carefully inject every embryo into the zona pellucida, avoiding penetration of the oocyte itself (*see* **Note 5**).
9. For retraction of the injection needle it might be helpful to first increase the negative pressure in the holding needle and then retract the needle (*see* **Note 6**).
10. Repeat the above steps with the remaining oocytes.
11. Harvest the oocytes after injection and transfer them into a new in vitro fertilization dish using a type III handling pipette.
12. Culture the embryos overnight at 37 °C.

3.7 Retransfer of Injected Oocytes into the Oviduct of Foster Mothers

3.7.1 Preliminaries

1. Narcotics: 10 % Ketamine; 2 % Xylazine.
2. Sterile surgery conditions and assorted surgical instruments: scissors, forceps, micro-serrefines, Dumont forceps, 2 mm blade spring-type micro-scissors; catgut.
3. Prepare sterile mounted swabs, disinfection spray, razors, and—if necessary—a big scissor to cut the hair at the flanks.
4. Prepare the surgical microscope with a 6× magnification.
5. Lay out handling (retransfer) pipette type IV and a pipette holder with mouthpiece.

3.7.2 Retransfer Procedure

1. The foster mothers should be pseudopregnant as verified by the presence of a vaginal plug in the morning immediately before conducting the retransfer.
2. Anesthesia is performed as described above. The recipient females are shaved laterally on both sides of the abdomen, between the thigh and the costal arch.
3. Disinfect properly and position the animal under a surgical microscope. The skin and the abdominal muscles are incised in a 1 cm transverse section approximately 2 cm caudal to the rips.

4. The fat tissue that becomes visible should be pulled out. The ovary can then be easily localized within the fat pad. Pull it out gently and place the ovary on the skin. Fix with microserrefines.
5. Apply one small drop of Suprarenine® (with the help of an insulin syringe) to the blood vessels of the membrane surrounding the ovary (*bursa*) to reduce bleeding (*see* **Note 7**). The anatomy of the ovary and oviduct is illustrated elsewhere (31). Carefully grab the bursa with a Dumont forceps and cut the membrane with a 2 mm spring scissor. Remove blood with a small sterile mounted swab. Identify the *infundibulum* and position it easily visible.
6. Collect 15–20 two-cell stage embryos with the handling (retransfer) pipette type IV. Aspirate two small bubbles in M16 medium, then aspirate the oocytes with only a minimal amount of surrounding medium. Afterwards, aspirate a third small bubble. The filling of the needle is illustrated elsewhere (31).
7. Insert the tip of the retransfer pipette carefully into the infundibulum of the oviduct. Once inserted, fix the *infundibulum* with Dumont forceps. Inject the embryos into the *infundibulum*.
8. After transfer, carefully reposition the ovary with the oviduct into the abdominal cavity. Suture the abdominal muscles and subsequently the skin. Repeat on the other side.
9. Postoperative analgesia should be provided for 10–14 days.

3.8 Follow-Up

Genotyping of the offspring should be performed at the age of approximately 3 weeks. We suggest marking the animals in a distinct and unfading fashion, for example using ear punches or tags.

Depending on the type of transgene vector used different genotyping procedures can be employed. We suggest isolating genomic DNA from tail tip biopsies and analyze it using a PCR strategy targeting at least two different regions of the transgene (*see* **Note 8**). In addition it might be reasonable to perform FACS analysis to detect expression of fluorochromes (e.g., eGFP) or surface markers included in the transgene construct (*see* **Note 9**).

Analysis of the phenotype is usually done after the first backcross to wild-type rats of the same strain (*see* **Note 10**). If germline transmission is confirmed, different lines from the founder rats are established and phenotyped.

The number of offspring that is obtained from each embryo transfer depends on the number of transferred embryos and their quality, and may occasionally reach up to 15 pups (*see* **Note 11**).

4 Notes

1. To control for the success of vasectomy, it is best to mate the vasectomized males with female rats of the same strain for at least 5 days. Within this period each female should be fertile for at least 1 day, which can be determined by smear pap tests. Within the fertile phase the rats will mate and a vaginal plug should be present. Without the presence of a plug the success of the vasectomy cannot be determined. To be absolutely sure we recommend keeping the females that had a plug for at least 3 more weeks. In case no pregnancy occurs and the pap test was negative for spermatozytes, it is safe to use the vasectomized males.
2. The calculated time points are based on a light/dark cycle from 6 a.m. to 6 p.m. If the conditions in your animal facility are different you should adjust the time schedule accordingly.
3. Use foster mothers only when a plug is present, since otherwise pseudopregnancy is not guaranteed, which might lead to the rejection of the transferred embryos. An otoscope can help to identify the plug which can be located deep in the vagina. In case insufficient numbers of female rats become plug-positive you might need to increase the number of matings next time or use other males.
4. Avoid long incubation of oocytes with hyaluronidase. Harvest the cells as soon as single cells become visible and the surrounding cumulus mass has sunken to the bottom of the well. If you have large numbers of oocytes we suggest performing consecutive treatments of smaller numbers of cells. The oocytes are much easier to handle if the membrane is still strong.
5. To inject the lentiviral particles, the injection needle with the constantly flowing solution is pierced through the *zona pellucida* into the *perivitelline space*. Then retract the needle a little bit. The membrane should expand visibly, but the oocyte itself should not.
6. From time to time you may observe that due to an increased diameter of the cell the oocyte sticks to the injection needle. In this case, apply a stronger negative pressure with the holding needle during and after the injection.
7. Small blood vessels are covering the ovary and one cannot avoid cutting through some of them. To prevent strong bleedings, apply a small drop of Suprarenine®. However, do not apply an excessive amount of Suprarenine® as this can interfere with the narcotics. If the bleeding is too strong to allow for location of the infundibulum you might have to extend the anesthesia by injecting an additional 50 µl of the two narcotics.

8. To make sure that the transgene vector is still intact after integration we suggest confirming its integrity with primer pairs corresponding to both ends of the construct.
9. Integration of the lentiviral transgene vector might occur in regions that are transcriptionally silent. Therefore it is not always guaranteed that transgenes detected by PCR will also be expressed on the mRNA and protein levels.
10. The first generation of transgenic rats must not be intercrossed to avoid double transgenic lines. Each founder should be mated with wild-type animals of the same background strain to make sure that putative multiple integrations will segregate in the offspring.
11. If no or only small litters are obtained it might be caused by toxic effects of the virus preparation or the quality of the cells. The majority of the one cell embryos should divide during overnight incubation before retransfer. If this is not the case we recommend leaving some oocytes uninjected in order to determine the influence of the virus preparation on the capacity of the oocytes to divide.

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DNA Vaccination Techniques

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Abstract

Multiple sclerosis (MS) is the most common inflammatory, demyelinating, and neurodegenerative disorder of the central nervous system (CNS) in humans. Although the etiology of MS remains unknown, several lines of evidence support the notion that autoimmunity against components of the myelin sheath plays a major role in susceptibility to and development of the disease. At present, there are no approved MS therapies aimed specifically toward downregulating antigen-specific autoreactive immune cells. One antigen-specific approach that appears promising for the treatment of MS is DNA vaccination. This technique has demonstrated efficacy in clinical trials while maintaining safety.

Here, we describe the generation of DNA vaccines containing immunologically relevant antigens of MS. Moreover, we present a detailed protocol for the prophylactic and therapeutic administration of DNA vaccines via intramuscular injection targeting on the development of experimental autoimmune encephalomyelitis (EAE), an animal model resembling MS.

Keywords: DNA vaccines, Multiple sclerosis, Experimental autoimmune encephalomyelitis, Proteolipid protein, Myelin basic protein, Myelin oligodendrocyte glycoprotein

1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system that mainly affects young adults between 20 and 40 years of age and leads to significant disability. The prevailing hypothesis to describe the pathogenesis of MS is that myelin destruction within the central nervous system (CNS) is due to antigen (Ag)-specific autoimmunity (1, 2). Although the majority of currently used drugs for MS treatment target immune responses, they are not selective for autoreactive T cells. Furthermore, while effective in some cases, current MS therapies may also alter host-protective immune responses. Ideally, treatment strategies in MS should aim to restore self-tolerance selectively to the pathogenic autoantigens while leaving the healthy immune system intact.

DNA vaccines represent a promising therapeutic approach for MS which also seem to overcome the safety concerns raised by other currently tested therapeutic strategies. DNA vaccination is a strategy of immunization based on the injection of genes encoding

for target proteins. Although DNA-based immunization research has largely focused on eliciting protective immunity against a variety of infectious pathogens, the technology may prove to have important applications in autoimmune diseases such as MS (3, 4). In this regard, DNA vaccines to treat autoimmune diseases should work as a "negative" form of vaccination, inducing tolerance rather than stimulation of immune responses to target antigens. DNA vaccines may induce immune tolerance by any number of potential mechanisms including anergy, clonal deletion, induction of T cells to differentiate to a less inflammatory effector phenotype or stimulating the production of regulatory cells (5). Thus, modulation or redirection of an antigen-specific cellular immune response resulting in a diminished inflammatory response may be beneficial. Vaccination using naked DNA encoding self-Ag has been shown to protect and even reverse established disease in several autoimmune animal models for various diseases, including rheumatoid arthritis (6), insulin-dependent diabetes mellitus (7) and MS (5).

Most of our current knowledge about new treatments in MS originates from experimental autoimmune encephalomyelitis (EAE) the animal model of MS, which mimics many clinical and neuropathological features of the disease (8, 9). In this model, the disease can be induced by immunization with components of the myelin sheath, and myelin peptides from the myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) have been identified as immunological antigens (10–12). Autoreactive T cells against these antigens are essential for the initiation and maintenance of the CNS inflammatory response leading to myelin destruction. Thus, different plasmid DNA vaccines expressing relevant sequences from each of the above-mentioned pathogenic autoantigens can be tested in order to induce tolerance or downregulation of the antigen-specific autoreactive immune responses.

In the following section, we report an example of effectiveness of DNA vaccines in ameliorating the clinical score of EAE. This chapter describes the construction of DNA vaccines encoding pathogenic myelin auto-Ag of MS (MBP, MOG, and PLP), the protocol to induce EAE in C57BL/6 by immunization with MOG peptide 35–55, and provides detailed protocols describing the preventive and therapeutic approaches of DNA vaccination used for the treatment of EAE. In addition, we evaluate the efficacy of the DNA vaccines in vivo and ex vivo. This chapter highlights the potential of DNA vaccination as a procedure to induce immune tolerance in autoimmune diseases. The work detailed here suggests that the technique is safe and effective and could be highly applicable to a clinical setting.

2 Materials

The molecular biology procedures used are basically as described in many molecular biology protocol manuals such as Green and Sambrook (2012) "Molecular Cloning: A laboratory manual (Fourth Edition)" and are not discussed in detail (13).

2.1 DNA Vaccine Construction

2.1.1 Isolation of Myelin Autoantigens from Mouse Brain

1. Mouse brain.
2. Round-bottom cryogenic vials (Nunc).
3. TRIzol reagent (Invitrogen).
4. High Capacity cDNA Archive kit (Applied Biosystems).
5. Accelrys DS gene 1.5 Software.
6. Oligonucleotide primers to amplify MOG, MBP, and PLP genes.
7. PCR amplification reagents (Promega): *Pfu* DNA polymerase, dNTPs, 10× buffer with MgSO4.
8. QIAquick PCR Purification Kit.

2.1.2 Development of Myelin-DNA Vaccines

1. Purified myelin antigens.
2. Plasmid: pCi mammalian expression vector (Promega).
3. Restriction enzymes: *EcoRI*, *NotI* and respective 10× buffers.
4. Mini-gel DNA electrophoresis apparatus, TAE, agarose, DNA stain, DNA size standards.
5. QIAquick Gel Extraction Kit (Qiagen).
6. UV protection mask, scalpel tip, UV or blue-light transilluminator.
7. T4 DNA ligase and 10× buffer.
8. MAX Efficiency® DH5α™ Competent Cells (Invitrogen).
9. Qiagen Endotoxin-free Mega kit (Quiagen).
10. Nanodrop ND-1000 (Thermo scientific).

2.2 DNA Vaccination of Mice

1. Purified DNA vaccines (MOG-, MBP-, and PLP-DNA) and control plasmid (pCi).
2. Endotoxin-free phosphate-buffered saline (PBS).
3. 2 ml tubes.
4. 22–25 gauge needles.
5. 1 ml syringes.
6. 27-gauge needles.
7. Female C57BL/6, 18–20 g (5–10-weeks old) (*see* **Note 1**).

8. Electric clippers.
9. Rodent isoflurane anesthesia machine.
10. Acrylic glass nebulizing chamber (approx. 25 × 25 × 25 cm).

2.3 EAE Induction

1. MOG residues 35–55 (MEVGWYRSPFSRVVHLYRNGK), at 90 % or greater HPLC purity (*see* **Note 2**). Obtain in lyophilized form and dissolve in sterile PBS at high concentration (10 mg/ml).
2. PBS.
3. Incomplete Freund's adjuvant (IFA) (Sigma).
4. Heat-inactivated, lyophilized Mycobacterium tuberculosis strain H37R (Difco).
5. 2 ml tubes.
6. Two 2 ml syringes connected by a 2-way luer lock adapter.
7. 1 ml syringes with removable 26-gauge needle.
8. Anesthetic solution: Ketamine (37 mg/kg)/Xilacine (5.5 mg/kg).
9. Electric heating pads.
10. Pertussis toxin (P.T.) from Bordetella pertussis (Sigma).
11. Mouse restrainer for intravenous injections.
12. Ear tags.
13. Petri dishes, polystyrene, size 60 mm × 15 mm.

2.4 Detection of Cellular Immune Responses

1. Mouse spleen.
2. Culture petri dishes, size 60 mm × 15 mm.
3. 5 ml syringes.
4. Tubes, 15 and 50 ml.
5. Red blood cell (RBC) lysing buffer.
6. PBS pH 7.4, 10×.
7. Full Dulbecco's medium supplemented with 10 % fetal bovine serum, 50 μmol/ml of 2-mercaptoethanol, 2 mmol/ml glutamine, 50 U/ml of penicillin and 50 mg/ml of streptomycin.
8. Trypan blue.
9. Hemocytometer.
10. Round-bottom 96-well plate.
11. MOG_{35-55} peptide.

3 Methods

3.1 DNA Vaccine Construction

3.1.1 Isolation of Myelin Autoantigens from Mouse Brains

1. Sacrifice C57BL/6 mice by CO_2 asphyxiation.
2. Immediately after death remove quickly the brain from skull and place it in round-bottom cryogenic vials and immersed in liquid nitrogen (*see* **Note 3**).
3. Isolate total mouse brain using the TRIzol reagent (Invitrogen) following the protocol for extraction of total RNA samples from animal tissues as recommended by the manufacturer.
4. Convert 1–2 μg of total RNA to cDNA using the High Capacity cDNA archive kit.
5. For primer design, first generate restriction maps of the mouse MOG, MBP, and PLP full-length genes and the recipient vector (pCi) using the Accelrys DS gene 1.5 Software. Add restriction sites to the primer sequences for the subsequent subcloning of genes into the pCi plasmid. We choose the restriction sites *EcoRI* (GAATTC) and *NotI* (GCGGCCGC) (*see* **Note 4**). The sequences of the forward and reverse primers used to amplify the MOG, MBP, and PLP genes by PCR are displayed in Table 1 (*see* **Note 5**).
6. Conduct PCR reactions using the following reagents: *Pfu* DNA polymerase, dNTPs, 10× buffers with MgSO4, and the corresponding forward and reverse primers. The PCR conditions are shown in Table 2.
7. Purify PCR products using the QIAquick PCR Purification Kit following the manufacturer's protocol.

Table 1
Primer sequences for generating myelin autoantigens

MOG Forward	5′-AAA**GAATTC**GATGGCCTGTTTGTGGAGCT-3′
MOG Reverse	5′-AAA**GCGGCCGC**CAGGAAGACACAACCAT CACTCA-3′
MBP Forward	5′-AAA**GAATTC**TAGCCTGGATGTGATGGCAT-3′
MBP Reverse	5′-AAA**GCGGCCGC**CAGGATTCGGGAAGGCTGA-3′
PLP Forward	5′-AAA**GAATTC**AAGTGCCAAAGACATGGGCT-3′
PLP Reverse	5′-AAA**GCGGCCGC**GCTCAGAACTTGGTGCCT-3′

All primers were designed with software Accelrys DS gene 1.5. The *EcoRI* and *NotI* restriction sites are shown in *bold*. The bases to fill at the 5′ end of the forward and the reverse primers are underlined (*see* **Note 5**)

Table 2
PCR conditions

MOG		MBP		PLP		
94 °C	5′	94 °C	5′	94 °C	5′	
94 °C 61 °C 72 °C	30″ 30″ 1′	94 °C 61 °C 72 °C	30″ 30″ 1′	94 °C 57 °C 72 °C	30″ 30″ 1′	35 cycles
72 °C	5′	72 °C	5′	72 °C	5′	

3.1.2 Development of Myelin-DNA Vaccines

1. Digest purified myelin antigens and pCi vector sequences with the restriction enzymes *EcoRI* and *NotI*.
2. Run the digestion products by agarose gel electrophoresis, and recover the fragments from the gel using the QIAquick Gel Extraction Kit, according to the user manual.
3. Set up ligation reactions: each insert is ligated into the pCi vector with T4 DNA ligase as described by the manufacturer.
4. Perform the transformation of DH5α™ Competent Cells, each ligation reaction per vial of *E. coli* DH5α.
5. Carry out a large-Scale DNA preparation using a Qiagen Endotoxin-free Mega kit.
6. Measure the DNA concentration of the purified DNA vaccines using a Nanodrop ND-1000.

3.2 DNA Vaccination

3.2.1 Mice and Experimental Setup

1. Mice: Female C57Bl/6 mice are chosen to induce the EAE model; C57BL/6 mouse displays chronic–progressive disease following active EAE induction with the MOG peptide 35–55 that resembles the later stages of MS. This chronic disease is characterized by sustained priming antigen-specific T cell responses, and it has been proved to be an important model to dissect regulatory and immunopathomechanistic pathways in EAE (14).
2. Experimental setup: Allocate mice to four groups (at least ten animals per group)—*see* **Notes 6** and **7**) and conduct DNA vaccination either with the myelin DNA vaccines (MOG, PLP, or MBP), or with the vehicle (empty pCi plasmid) as control. For prophylactic schedule, mice are injected intramuscularly at 4 and 2 weeks before EAE induction. For therapeutic treatment, mice receive intramuscular (i.m.) injections of DNA at disease onset (day 10 post EAE induction) and a second dose 2 weeks later.

3.2.2 Vaccine Administration

1. Prepare 100 μg of each plasmid DNA in a volume of 100 μl endotoxin-free PBS per mouse and transfer from the vial into a 1 ml syringe using a 22–25 gauge needle.
2. Shave the flank of the animals to be treated with electric clippers. Clean the area with ethanol to ensure full removal of oil, dust, and dander.
3. Anesthetize the animals with inhaled isoflurane (5 %) in an acrylic glass nebulizing chamber and maintain light anesthesia with isoflurane (3 %) during the treatment by placing the snout into an inhalation tube.
4. Inject each mouse with a total volume of 100 μl of plasmid DNA (1 μg/μl), by two i.m. injections with 50 μl into tibialis anterior muscle of both hind legs with a 1 ml syringe using a 27 gauge needle.

3.3 EAE Induction

3.3.1 Preparation of Emulsion of Antigen and Adjuvant for Immunization

1. Estimate the amount of peptide and CFA for the immunization: according to our experience using the EAE model, immunization of mice with 50 μg of the MOG_{35-55} peptide in 200 μl of an emulsion with CFA is sufficient for the reproducible induction of EAE at high incidence.
2. Emulsion volume: Each mouse receives 200 μl of emulsion. To calculate the volume, consider in excess because there is a waste during the emulsification process (e.g., for 10 mice, instead of 2 ml prepare 2.4 ml of emulsion, calculated considering 12 mice).
3. Emulsion composition: Each mouse receives 50 μg of MOG_{35-55} in complete Freund's adjuvant (CFA), that is incomplete Freund's adjuvant (IFA) supplemented with 4 mg/ml of Mycobacterium tuberculosis.
4. Prepare the emulsion: The solutions of MOG in PBS (0.5 mg/ml) and Mycobacterium tuberculosis in IFA (4 mg/ml) are prepared separately, in two different 2 ml tubes, and then mixed in a 1:1 volume ratio (usually we prepare emulsion for 10 mice: 2.4 ml of emulsion: 1.2 ml of 0.5 mg/ml MOG in PBS and 1.2 ml of 4 mg/ml Mycobacterium tuberculosis in IFA). Mix CFA and peptide solution by repetitive passage between two glass syringes connected by a stopcock after removing as much air from the two syringes as possible. Emulsify for 6–8 min, until the solution is firm enough (*see* **Note 8**).
5. Load plastic syringes with emulsion: Pump all of the emulsion into one of the 2 ml syringes. Remove the empty 2 ml syringe from the stopcock and replace it with a 1 ml syringe. Slowly transfer 600 μl emulsion (enough for three mice) without air, and add to the syringe a 26-gauge needle. Repeat until all of the emulsion is dispensed into plastic syringes.

Table 3
Scale used for clinical evaluation

0	Healthy
1	Limp tail only. The tail remains flaccid when the mouse is picked up.
2	Hind limb paresis, but without frank leg dragging
3	Complete hind limb paralysis
4	Paraplegia with forelimb weakness or paralysis
5	Moribund state or death

3.3.2 Induction of EAE

1. Anesthetize mice with 50 μl/20 g body weight with a combination of Ketamine (37 mg/kg) and xylazine (5.5 mg/kg), via intraperitoneal injection using a 27 G syringe.
2. As soon as the mouse is in deep anesthesia (*see* **Note 9**) you can proceed to identify mice by ear punch.
3. Hold the mice from the neck and the tail and inject subcutaneously (s.c.), slowly, with 200 μl of emulsion under the skin of both flanks (100 μl at each site) (*see* **Note 10**).
4. Place the mice on electric heating pads to prevent excessive heat loss (*see* **Note 11**) and monitor the mice till they are fully awake. The mice typically are fully awake within 30–40 min.
5. After immunization, mice receive 150 ng of P.T. resuspended in 100 μl PBS in the tail vein. On day 2 post immunization (p.i.) inject the mice again with 150 ng of P.T. Mice are held in a restrainer for i.v. injection.

3.4 EAE Clinical Evaluation

1. Clinical score and body weight is recorded daily.
2. Clinical onset usually occurs, variably, between the 7th and 12th day after immunization.
3. A nonparametric scale with five steps is used for clinical evaluation (Table 3)
4. Please refer to Notes 12–15 for further suggestions.

3.5 Efficacy of Prophylactic or Therapeutic DNA Vaccination in Chronic-Progressive EAE

C57BL/6 mice immunized with MOG_{35-55} suffer chronic progressive EAE with onset around day 7. MOG-DNA vaccination ameliorate the clinical signs of the disease when administered according to a prophylactic schedule, as well as to a therapeutic schedule, starting at disease onset (Fig. 1) compared with the control plasmid group (pCi). Prophylactic MOG-DNA treatment also delayed disease onset [mean (SEM): 10.6 days (0.7) in MOG-DNA treated mice vs. 7.5 days (0.3) in control plasmid-treated

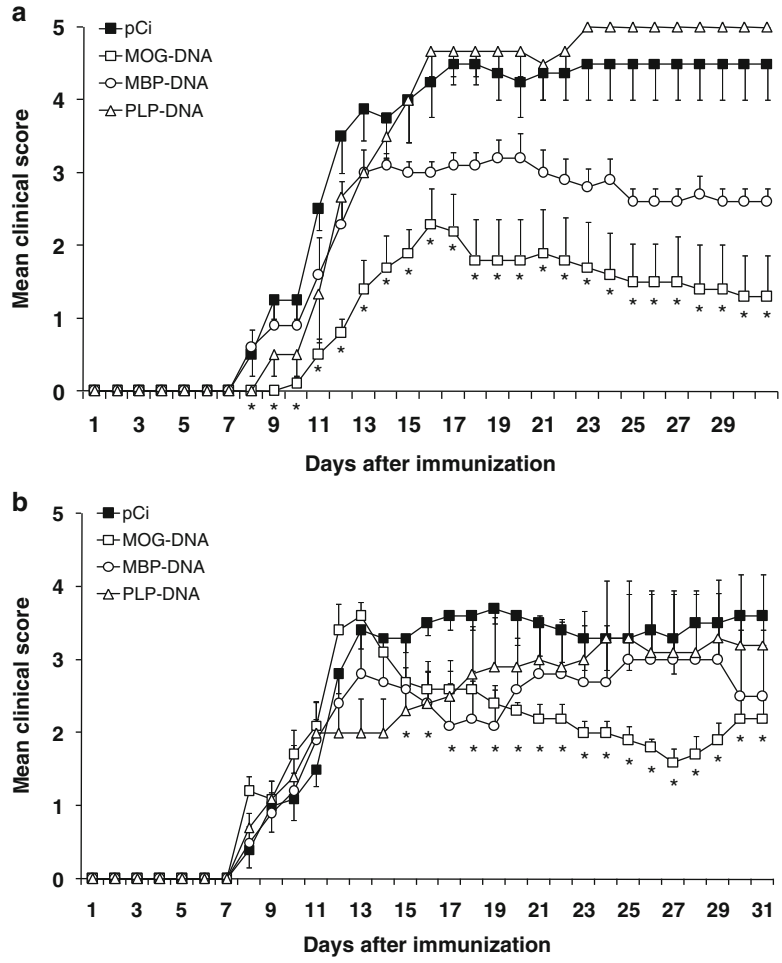

Fig. 1 Prophylactic and therapeutic MOG-DNA treatment improves the clinical course of EAE. EAE was induced in female C57BL6/J mice with MOG_{35-55} peptide in CFA. Mice were vaccinated with i.m. injections of 100 μg of DNA according to prophylactic (**a**) or therapeutic (**b**) protocols as described in Section 3. Four groups of mice in each of the scheduled treatments received the full-length MOG-DNA construct (*open square*), MBP-DNA (*open circle*), PLP-DNA (*open triangle*), or the plasmid control, pCi (*filled square*). Mean clinical scores are plotted against the number of days after EAE induction. Disease scores are expressed as mean values (SEM). Statistically significant differences obtained by Student's t-tests are denoted with asterisks (*$p < 0.05$)

mice; $p = 0.006$], and reduced clinical severity [mean cumulative clinical scores: 1.4 (0.4) vs. 3.8 (0.3) in MOG-DNA-treated and control plasmid-treated mice respectively; $p = 0.003$] (Fig. 1a). MOG-DNA treatment improved ongoing EAE, as reflected by the significant reduction in the mean cumulative EAE clinical score observed in MOG-DNA-treated mice compared with pCi-treated mice [2.2 (0.1) vs. 3.0 (0.3); $p = 0.031$] (Fig. 1b). The beneficial effect observed after MOG-DNA prophylactic and

therapeutic vaccination of mice with MOG-induced EAE is antigen-specific since neither MBP-DNA (reduction in disease severity, although to a lesser degree compared with MOG-encoding DNA vaccine) nor PLP-DNA vaccination are associated with a significant reduction in MOG-induced EAE compared with the control group (Fig. 1).

3.6 Detection of Cellular Immune Responses Ex Vivo

3.6.1 Collection of Spleens and Single-Cell Suspension Preparation

1. On day 14 after EAE induction, sacrifice five mice per group of prophylactically DNA-treated animals (MOG-DNA and pCi) and remove spleen in aseptic conditions using sterile scissors and forceps.
2. Place the spleen into 100-mm cell culture plate containing 10 ml sterile 1× PBS.
3. Disrupt the spleen tissue by pressuring using the syringe column. Collect cell suspension from the plate in 15 ml tube.
4. Spin down cell suspension at 400 × *g* for 5 min.
5. Remove the supernatant and resuspend the pellet of splenocytes with 5 ml of RBC lysis buffer for at least 5 min at room temperature.
6. Add 10 ml 1× PBS and centrifuge at 400 × *g* for 5 min.
7. Discard supernatant and wash pellet two more times with 10 ml 1× PBS.
8. After the last washing step discard supernatant and resuspend cell pellet in 10 ml Dulbecco's media.
9. Count cells stained with trypan blue by automated cell counter or using hemocytometer.
10. Resuspend lymphocytes to a concentration of 4×10^6 cells/ml.

3.6.2 Determination of Cytokine Levels of IFN-γ and IL-17 (15, 16)

1. Culture splenic-single cell suspensions from mice in a round-bottom 96-well plate.
2. Seed spleen cells at a final concentration of 2×10^5 cells/well in a total volume of 200 μl of full Dulbecco's medium with 10 μg/ml of MOG_{35-55} as stimulus.
3. Cells cultured without stimulus are used as baseline controls.
4. Incubate cultures in a humidified atmosphere at 5 % CO_2 and 37 °C for 72 h.
5. After stimulation, collect supernatants in 2 ml tubes and determine the cytokines levels of IFN-γ, IL-10, IL-4, and IL-17 with the FlowCytomix kit according to the manufacturer's recommendations.

4 Notes

1. We usually induce EAE in mice 9–10 weeks old. Thus, use 5–6-week old animals for prophylactic DNA vaccination (scheduled doses of DNA at 4 and 2 weeks previous EAE induction) and 9–10-week old for therapeutic DNA vaccination.
2. Quality of the peptide is a key issue to obtain a good disease. Incomplete incidence or very low severity of EAE is related to peptide's quality.
3. To preserve RNA integrity, snap-freeze the tissue very rapidly after dissection. Samples can be stored at −80 °C for later use.
4. Sites cannot be added to the ends of the insert region if they exist within the insert sequence, because the PCR fragment will need to be cut with those enzymes before cloning.
5. The bases at the 5′ end of the forward and the reverse primers are just filler to ensure efficient digestion close to the end of the fragment.
6. House animals in groups or individually based on intuitional cage size with ad lib access to food and water. Randomly assign mice to treatment groups. Allow acclimatization for 5 days.
7. To calculate the amount of animals needed for an EAE experiment, we have to take into account the incidence of the disease (disease incidence for EAE induced with MOG_{35-55} in C57BL/6 mice is in order to 90 %) and the mortality rate (between 10 and 20 %). Thus, we use the following formula:

   $X = N/(A/100 \times B/100 \ldots \text{etc}) =$ Initial number of animals ($N =$ minimum number of animals for statistical significance, $A =$ % disease incidence, $B =$ % mortality)
8. At periodic intervals test the emulsion by placing a drop in a Petri dish containing PBS. The emulsion is ready when a small drop placed on the surface of PBS does not disperse instantly but maintains its integrity.
9. In order to test if the mouse is in deep anesthesia, you can perform a paw pinch test. If no reflex is present, you can proceed.
10. Since the emulsion is very dense, a great pressure has to be applied to push the needed amount out of the syringe. This can lead to the popping off of the needle, and sometimes the emulsion comes out with great pressure and can get in the operator's eyes. Therefore, it has to be pressed firmly when put the 26-gauge needle back to the 1-ml syringe.
11. Hypothermia induced by anesthesia may alter physiological processes that can even result in mice death (17). Therefore

after anesthesia we keep mice warm using electric heating pads to prevent excessive heat loss during anesthetic procedures.

12. The clinical follow-up is carried out blinded by a single researcher.
13. We daily weigh the mice and we use that as "surrogate marker" of EAE.
14. When we have mice with a clinical score of 3 or 4 (not able to move properly). We place food pellets and water in a small petri dish (polystyrene, size 60 mm × 15 mm) into the cage to enable sick mice to eat and drink.
15. We sacrifice mice before they progress to a score of 5.

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Part II

Cellular Techniques

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Peripheral Blood Mononuclear Cells: Isolation, Freezing, Thawing, and Culture

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Abstract

The work with peripheral blood mononuclear cells (PBMCs), which comprise lymphocytes and monocytes, is indispensable in immunological diagnostics and research. The isolation of PBMCs takes advantage of differences in cell density of the different blood components. Density gradient centrifugation of diluted whole blood layered over a density gradient medium yields PBMCs; two subsequent washing steps remove remaining platelets. To store the cells for future assays, they can be frozen and thawed when required. Dimethyl sulfoxide (DMSO) serves as a cryoprotectant for freezing PBMCs, but must be removed by washing after thawing, as it can become toxic to the cells on longer exposure.

Keywords: PBMC, Density gradient, Freezing, Thawing, Cryopreservation

1 Introduction

The investigation of the human immune system is a broad field of research and the key to understanding many different diseases and their possible future cure or prevention. Immunological research contributes to deciphering the etiology and pathogenesis of autoimmune diseases like multiple sclerosis, to characterizing the role of immune cells during infections, to the development of vaccines and to a better understanding of the pathogenesis and possible future therapies of malignancies. For analysing adaptive immune responses, a starting point and fundamental method is the isolation of peripheral blood mononuclear cells (PBMCs) from whole blood. PBMCs comprise lymphocytes and monocytes. In subsequent experiments, they can be further separated, for example by magnetic cell separation or fluorescence-activated cell sorting. Lymphocytes and monocytes can also be employed together in assays like ELISPOTs or proliferation assays, where antigen-presenting cells are necessary to present antigenic peptides on MHC-I or MHC-II to the responding T cell populations.

Often it is not possible to work with freshly isolated PBMCs. Then the cells can be frozen and thawed at a later time point. This allows a batched thawing of different samples and a direct comparability in assays, reducing inter-assay variability, as well as a future analysis of later emerging issues. Many studies have been conducted exploring the viability and antigen-specific response of cryopreserved mononuclear cells, most of them in the context of HIV research. It has been shown that cryopreservation does not functionally impair cell cycle or cytokine-production of ($CD4^+$ and $CD8^+$) T cells upon stimulation (1, 2). Percentages of $CD4^+$ and $CD8^+$ cells could be shown to be equal in fresh and cryopreserved PBMC and were also stable in cryopreserved cells for more than a year (3, 4). However, it must be taken into consideration that cryopreservation can reduce the fraction of naïve and central memory T cells, can lead to a decrease of CCR-5 expression and to an increase of effector $CD8^+$ cells (4). All in all, a good quality of assays with cryopreserved cells can be maintained.

The isolation of PBMCs from whole blood is based on density differences between PBMCs and other components of the peripheral blood. The origins of this method go back to the 1960s when Arne Boyum first described the separation of white blood cells using Ficoll and other density gradient media (5). Since then, the technique has been refined, but the underlying principles have remained the same: By density gradient centrifugation, a separation of blood components according to their density is possible with the help of a density gradient medium (e.g. Lymphoprep or Ficoll) containing sodium diatrizoate, polysaccharides, and water, reaching a density of 1,077 g/ml. The density gradient medium leads to an aggregation of red blood cells. The medium is therefore denser than lymphocytes, monocytes, and platelets, but less dense than granulocytes and red blood cells. Accordingly, erythrocytes and most of the granulocytes will sediment and pellet at the bottom of the tube after centrifugation; over this phase, the density gradient medium is found. The top layer consists of plasma and platelets. Mononuclear cells band at the interface between the plasma and the density gradient medium. Two subsequent washing steps at a lower speed help to remove remaining platelets.

For cryopreservation, dimethyl sulfoxide is widely used as a cryoprotectant. It reduces the formation of ice crystals and resulting cell damage. Despite its benefits, it can be toxic to cells (6). When PBMCs are thawed the comprised DMSO should therefore be quickly diluted and removed by two subsequent washing steps. In the following, we describe how PBMCs can be isolated, cryopreserved, and thawed. In addition, we point out some basic principles for culturing PBMCs.

2 Materials

All materials and reagents coming into contact with cells must be sterile. Keep attention that your plastic material, reagents, and media are not only sterile, but also free of endotoxin. Store all reagents as indicated.

1. 1× Phosphate Buffered Saline (PBS).
2. 50 ml tubes.
3. Sterile plastic pipettes (*see* **Note 1**).
4. Transfer pipettes.
5. Lymphoprep: 9.1 % w/v Sodium Diatrizoate, 5.7 % w/v Polysaccharide, water. Density of 1.077 g/ml (Stemcell Technologies, Oslo, Norway).
6. 1× RPMI 1640 Medium (*see* **Note 2**).
7. Cell medium: 10 % Fetal Bovine Serum (FBS) (*see* **Note 3**), 1 % 200 mM L-Glutamine and 1 % Penicillin/Streptomycin in RPMI 1640 medium.
8. 0.4 % Trypan blue: Make a 0.4 % trypan blue solution by weighing the requested amount of trypan blue powder and diluting it with PBS (*see* **Note 4**).
9. Neubauer Counting Chamber.
10. DMSO (*see* **Note 5**).
11. Freezing medium: 80 % FBS and 20 % DMSO.
12. Cryogenic Vials: Vials must be sterile and resistant against liquid nitrogen.
13. 96-well or 24-well plastic plates.
14. Phytohemagglutinin.

3 Methods

Take care that everything coming into contact with cells remains absolutely sterile.

3.1 Isolation of PBMCs

1. Dilute the blood (*see* **Note 6**) with a double volume of PBS.
2. Prepare 50 ml tubes filled with 15 ml of Lymphoprep each. You need one tube per 10 ml whole blood or 30 ml blood dilution, respectively.
3. Carefully layer 30 ml of the blood dilution upon the Lymphoprep solution. Avoid an intermingling of the two phases (*see* **Note 7** *and* Fig. 2).

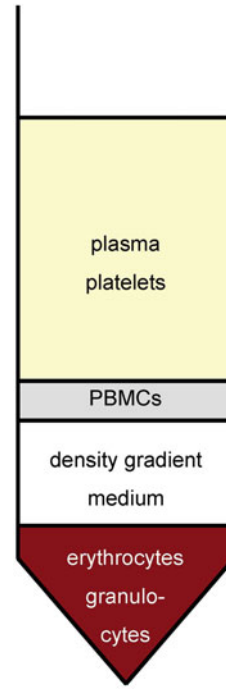

Fig. 1 After density gradient centrifugation, different blood cells are found in different layers according to their density. From bottom to top you find red blood cells and granulocytes, followed by the density gradient medium (e.g. Lymphoprep), the layer containing the PBMCs and on top the plasma phase containing platelets

4. Centrifuge for 25 min at 670–800 × *g* at 18 °C (*see* **Note 8**). Make sure the centrifuge brake is switched off to prevent a disruption of the gradient layers.
5. Collect the cloudy-looking phase containing the PBMCs, which you find above the Lymphoprep layer and beneath the phase containing plasma and platelets (*see* Fig. 1 *and* **Note 9**). Transfer PBMCs of three 50 ml tubes into one new 50 ml tube. Add PBS to make up to 50 ml.
6. Centrifuge for 10 min at 150 × *g* at 4 °C with the centrifuge brake switched off.
7. Decant the supernatant, resuspend the pellet in RPMI medium and centrifuge again for 10 min at 150 × *g* and 4 °C with the centrifuge brake off (*see* **Note 10**).
8. Decant the supernatant and resuspend the resulting pellet in cell medium (*see* **Note 11**).
9. Count the cells (*see* **Note 12**).
10. Freeze the cells or perform an assay with the freshly isolated cells.

3.2 Cryopreservation of PBMCs

1. Prepare cryovials and label them with cryotags or with a permanent marker (*see* **Note 13**).
2. Define the concentration at which you want to freeze the PBMCs and the quantity of cells contained in one aliquot. Bring the cell suspension to the double concentration of your end concentration by adding cell medium.
3. Add an equal volume of freezing medium in three subsequent steps in intervals of 3–4 min to give your end concentration (*see* **Note 14**).
4. Mix the cell suspension and transfer aliquots of the PBMCs to the cryovials.
5. Freeze the cells in a −80 °C freezer as fast as possible.
6. Transfer the cells to liquid nitrogen or to a freezer providing temperatures lower than −150 °C within 2–3 days. Store them at this low temperature until use.

3.3 Thawing PBMCs

1. Warm RPMI medium in a water bath at 37 °C for washing your thawed cells in (*see* **Note 15**).
2. Thaw the cells in the water bath (*see* **Note 16**).
3. Quickly resuspend the cells in the medium and centrifuge at 150 × *g* for 10 min at 4 °C with the centrifuge brake off.
4. Decant the supernatant, resuspend the cells in RPMI and wash again as in step 3 (*see* **Note 17**).
5. Resuspend the cells in cell medium and count them.

3.4 Culturing Cells

1. For culturing the PBMCs, bring them to the desired concentration by diluting with cell medium.
2. Add the cells to 96-well plates or 24-well plates (*see* **Notes 18–21**).
3. Incubate the cells at 37 °C and 5 % CO_2 in an incubator.

3.5 Stimulating Cells

1. When you perform an assay with PBMCs and stimulate them with different antigens, always introduce positive and negative control wells (*see* **Note 22**).
2. Pre-establish the optimal concentrations of your stimuli in titration experiments.
3. After the incubation time of your assay has ended, collect the supernatant and freeze it at −80 °C. You can later perform multiplex analyses to determine the prevalent cytokine milieu in different stimulatory situations.

4 Notes

1. It can be highly recommended to use one-way plastic pipettes in immunological settings. Lipopolysaccharide, when sticking to glass pipettes, can withstand autoclaving and stimulate cells coming into contact with it.
2. Cell media contain phenol red, a pH indicator. As the medium contains 5 % CO_2, the pH rises when the contact with ambient levels of CO_2 increases. In case the medium turns pink it should be discarded.
3. Do not use untested serum. Even small quantities of contaminants like stimulators (e.g. bacterial endotoxin) or suppressors can ruin the result of possible subsequent assays. Stick to the use of one serum and always use serum of one batch, if possible.
4. Trypan blue is cancerogenic. Avoid skin contact when weighing the trypan blue powder. Trypan blue does not need to be handled sterilly, but it is recommended to add sodium acid or to filter the solution from time to time to avoid a growth of fungi which can obscure your view when counting cells.
5. DMSO is combustible and irritating to eyes, respiratory system, and skin. It is absorbed through the skin. Avoid contact.
6. Try to draw the blood as carefully as possible. We can recommend the use of sodium heparin as an anticoagulant, but other anticoagulants like acid citrate dextrose (7) or lithium heparin can be used. Do not chill the blood during the transport to the laboratory. It was found that a cryopreservation of PBMCs within 8 h is significantly superior to a cryopreservation within 24 h, which can reduce the viability and functional activity of your cells (7). Our experience is that it is realistic to freeze the cells within 4 h after venipuncture, which we observe has positive effects on cell viability.
7. The blood can be layered over the Lymphoprep medium in two ways:
   (a) Hold the tube containing the Lymphoprep solution at a tilted angle. Use a pipette for taking up the blood and make sure the pipetting speed is set to a minimum. Place the tip of the pipette against the wall of the tube (*see* Fig. 2b).
   (b) Prepare the same number of tubes as the ones filled with Lymphoprep and pipette 30 ml of the blood dilution into each. If you are right-handed, take a tube containing Lymphoprep in your left hand and a tube containing blood dilution in your right hand. Hold both tubes nearly horizontally and make the winding of the blood tube meet the rim of the Lymphoprep tube. Layer the blood dilution over the Lymphoprep medium by pouring the blood dilution very carefully into the Lymphoprep tube (*see* Fig. 2c).

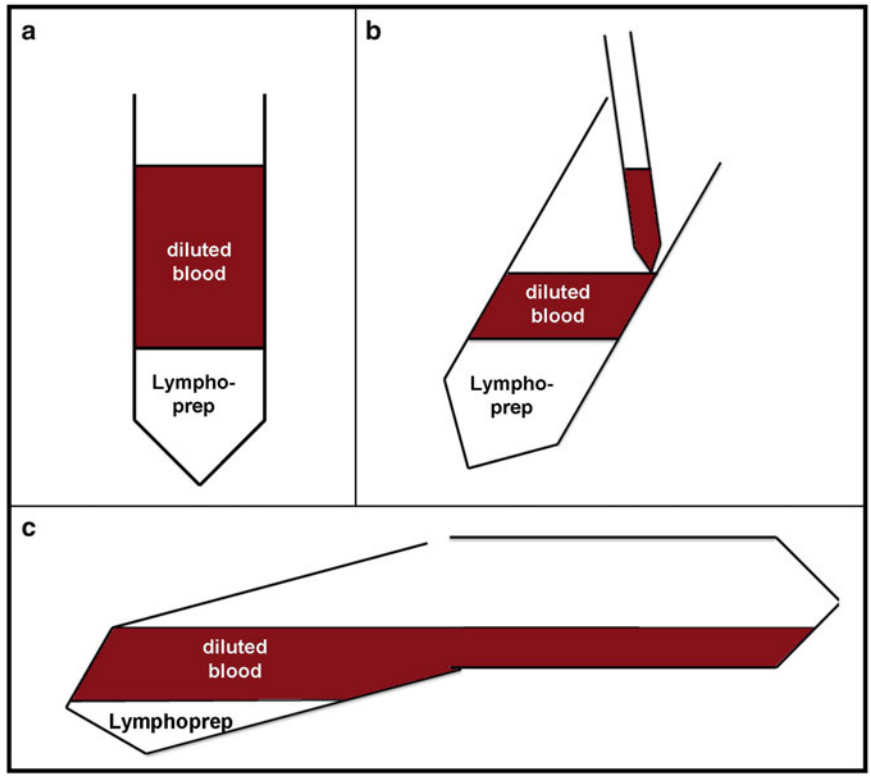

Fig. 2 This shows how the diluted blood can be layered over the Lymphoprep phase. (**a**) The two phases should not be intermingled. There should be a clear contour between the phases. (**b**) A pipette can be used for achieving this or (**c**) the blood dilution can be poured carefully from one tube into the other upon the Lymphoprep phase

8. Centrifugation at higher temperatures than 18–20 °C leads to lower cell yields, as more lymphocytes become trapped between aggregated erythrocytes. Lower temperatures increase the density of the density gradient medium and can cause a contamination of the PBMC layer with red blood cells and granulocytes.
9. It is best to use a transfer pipette for collecting the layer containing the PBMC. In contrast to bigger plastic pipettes, it is easier to avoid collecting either little volumes of the Lymphoprep phase or the plasma phase, leading to a contamination with granulocytes or platelets, respectively. Hold the tube so that the PBMCs are at eye level. So you can see best if the pipette remains in the right phase. Dispose of the tube containing the rest of the blood cells in a biohazard waste container.
10. If no pellet is visible after the centrifugation of the collected PBMC layer, it is recommendable not to discard the supernatant, but to decant it into new sterile falcon tubes, to add some

PBS and centrifuge it again in the next round. If there forms a pellet, you have saved the mononuclear cells you would have lost otherwise.

11. Normal PBMC yields range from 0.5 to 2.5 $\times$ 10^6 PBMCs per milliliter whole blood. If you freeze the cells afterwards, the quantity of cell medium you resuspend your cells in depends on the cell numbers frozen in one aliquot. For resuspending the cell pellet, we use about 5 ml cell medium per 100 ml starting blood volume and freeze aliquots of 5–10 $\times$ 10^6 PBMCs.
12. Mix the cell suspension thoroughly, then take about 10–20 µl of the cell suspension and transfer it to a cell culture plate. Mix it with an equal volume of trypan blue and dilute about 16–32-fold. Sometimes the gradient may be contaminated with some red blood cells. This can e.g. be due to problems at drawing the blood. When you count your cells with the trypan blue solution, you cannot discriminate between viable red blood cells and mononuclear cells, but the ratio of viable cells in all cells can be determined. Counting the cells again with Türk's solution reveals the number of mononuclear cells, as Türk's solution destroys red blood cells, but allows no discrimination between viable and apoptotic cells. Using both systems, the number of viable mononuclear cells can be estimated.
13. We prefer the use of printed cryotags, since their labeling withstands alcoholic disinfection.
14. A stepwise addition of DMSO helps the PBMCs to "get accustomed" to the cryoprotectant. However, the cells should best be frozen within 10 min. Make sure you have opened all the cryovials before you add the last portion of the freezing medium so that you can start aliquotting immediately.
15. It has been shown that adding cold medium rapidly to cold cells after thawing reduces the viability of the cells (8, 9). However, the volume of your thawing medium (e.g. 15 ml vs. 50 ml) does not influence cell viability (9).
16. Disinfect cryovials after taking them out of the water bath. Water baths are very susceptible to contamination.
17. Washing twice after thawing increases the viability of cells, since DMSO is diluted (8). The influence of DMSO on cell viability and functionality is dependent on the duration of exposure and concentration (10).
18. It is essential you have equal cell numbers in each well for good results of your assay. Make sure to mix your cell suspension thoroughly.
19. For transferring cells to a 96-well plate, you can use a multipette or a multichannel pipette. If you prefer a multichannel pipette, first transfer your cells into a sterile reagent reservoir.

20. There are different types of plates with U-shaped, V-shaped, and flat-bottom wells. Use plates with U-shaped or V-shaped wells for culturing cells when much cell-cell interaction is required. Use flat-bottom plates when you culture adherent cells.
21. Keep in mind that monocytes strongly adhere to plastic when you harvest your cells after culture.
22. As negative controls use wells containing PBMCs without stimuli. It is advisable to add the solvent of your stimuli (e.g. DMSO) to negative wells in the same concentration as in wells containing antigen. For positive control wells, you can add mitogens like phytohemagglutinin.

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ELISPOT Techniques

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Abstract

The enzyme-linked immunospot (ELISPOT) assay is a widely used method for enumerating antigen-specific cytokine-producing or antibody-secreting immune cells. It is one of the most effective immunological and diagnostic approaches to detect and quantify low-frequency cytokine- or antibody-producing cells in human and animal tissues, such as peripheral blood, lymph nodes, and spleen. Detection and quantification of specific cytokine-producing cells by the ELISPOT assay is based on the formation of visible spots at the site of cytokine release by the cells under investigation (e.g., T cells) using pairs of different capture and detection antibodies under optimized conditions.

Here we focus mainly on practical, optimized protocols for cytokine ELISPOT assays for detection of mouse and human cytokine-producing immune cells (e.g., peripheral blood mononuclear cells, PBMC), including suggestions for trouble-shooting and optimizing steps for problematic tissue samples.

Keywords: ELISPOT, Antigen recall, Cytokine, Antibody, Immune cells, T cells, PBMCs

1 Introduction

The ELISPOT assay was originally developed and reported in the early 1980s for enumerating specific antibody-secreting B cells (1–3). Subsequently, this technique was adapted and applied to quantify specific cytokine-producing immune cells from human or mouse samples (4–6). Since then, the ELISPOT technique has rapidly evolved and, in combination with ELISPOT image analysis, has been widely used in immunology research and clinical applications for measuring antigen-induced or spontaneous specific cytokine responses of many types of immune cells to microbial pathogens, vaccines, and foreign- or self-antigens (7–20). Cytokine ELISPOT is based on the principle that cytokines secreted by inflammatory cells are trapped by a capture antibody on a membrane and visualized by a secondary detection antibody in combination with a substrate that precipitates at the site of the captured cytokines to form visible spots (Fig. 1). In comparison to enzyme-linked immunosorbent assay (ELISA), the ELISPOT assay has the advantage of not only measuring the cytokine response at the single-cell level, but also being able to detect

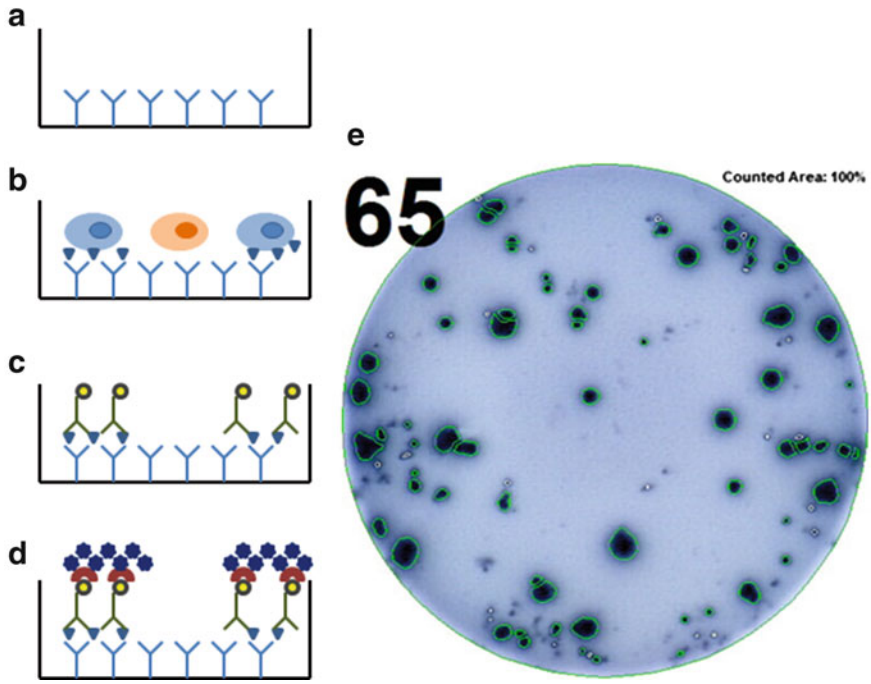

Fig. 1 Principle of ELISPOT. For cytokine ELISPOT assay, the membrane of an ELISPOT plate is coated first with capture antibody as shown in (**a**). (**b**) Cytokines that are produced by cells of interest (e.g., T cells) with or without specific stimulation (antigen or mitogen) during culture will bind specifically to the plate-bound capture antibody. (**c**) After the cells are removed, biotin-conjugated detection antibody will be added and binds to the captured cytokines. (**d**) Upon subsequent addition of streptavidin-enzyme conjugate and corresponding substrate, precipitates will form around the site of the antibody-cytokine-antibody complex and appear as visible spots, as shown in (**e**) in the case of BCIP/NBT substrate. (**e**) Example of a well from a developed and analyzed mouse-IFN-γ ELISPOT plate using day 9 draining lymph node cells restimulated with specific antigen

specific cytokine-producing cells with much higher sensitivity, even when the cytokine secreted into the supernatant is below detection level by ELISA (6, 21).

2 Materials

2.1 Capture and Detection Antibodies (See Note 1)

1. Mouse cytokine ELISPOT: use suggested clones for each monoclonal antibody (mAb) pair and the optimal working concentration or titration range listed in Table 1.
2. Human cytokine ELISPOT: use suggested clones for each mAb pair and the optimal working concentration or titration range listed in Table 2.

2.2 Medium and Buffers

1. 1× PBS, sterile and non-sterile.
2. Blocking buffer: sterile PBS with 1 % BSA (Sigma Aldrich).
3. Serum-free medium:

Table 1
Recommended antibody pairs for mouse cytokine ELISPOT assay

Cytokine	Capture mAb	Detection mAb (biotin-conjugated)
IFN-γ	AN-18 (1–2 μg/ml)	R4-6A2 (0.25–0.5 μg/ml)
IL-2	JES6-1A12 (4 μg/ml)	JES6-5H4 (0.5–1 μg/ml)
IL-4	11B11 (4 μg/ml)	BVD6-24G2 (2 μg/ml)
IL-5	TRFK (5 μg/ml)	TRFK4 (0.5 μg/ml)
IL-13	eBio 13A (2–4 μg/ml)	eBio 1316H (0.25–1 μg/ml)
IL-17	17 F3 (2 μg/ml)	TC11-8H4 (0.125 μg/ml)
GM-CSF	MPI-22E9 (2 μg/ml)	MP1-31G6 (0.25 μg/ml)

Listed are clone names and suggested concentration/titration range of both capture and detection mAbs for common mouse cytokines. Antibodies are available from several commercial sources including eBiosciences, Biolegend, and BD Biosciences

Table 2
Recommended antibody pairs for human cytokine ELISPOT assay

Cytokine	Capture mAb	Detection mAb (biotin-conjugated)
IFN-γ	2G1 (4 μg/ml)	B133.5 (0.75 μg/ml)
IL-17	eBio64CAP17 (2 μg/ml)	eBio64DEC17 (0.75 μg/ml)
IL-2	MT2A91/2C95 (5–10 μg/ml)	MT8G10 (0.25 μg/ml)
TNF-a	TNF3/4 (10–15 μg/ml)	TNF5 (0.5 μg/ml)

Listed are clone names and suggested concentration/titration range of both capture and detection mAbs used for common human cytokines. Antibodies are available from several commercial sources including Thermo Scientific, eBiosciences, and Mabtech

For mouse cytokine ELISPOT: HL-1 medium (Lonza, BioWhittaker).

For human cytokine ELISPOT using PBMCs or T cell line: CTL-Test Medium (Cellular Technology Limited, CTL).

Medium must be supplemented with 1 % L-glutamine (2 mM) and preferably prepared fresh, since the half-life of L-Glu is only 7–10 days at 4 °C.

4. PBST: 1× PBS containing 0.05 % Tween-20.

2.3 ELISPOT Plates, Enzyme, and Substrate

1. Millipore MultiScreen® sterile IP plates with high protein binding membrane (Millipore, Cat no. MSIPS4W10) are recommended for both human and mouse cytokine ELISPOT assays.
2. Streptavidin alkaline phosphatase conjugate (e.g., Life technologies/Invitrogen, Cat no. S-921, as used in author's

laboratory) or streptavidin-horseradish peroxidase (HRP) if 3,3′,5,5′-Tetramethylbenzidine (TMB) or 3-Amino-9-Ethylcarbazole (AEC) substrate will be used (e.g., BD Biosciences).

3. Substrate: 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) Phosphatase pre-mixed solution (e.g., Kirkegaard & Perry Laboratories, KPL, Cat no. 50-81-08, as used in author's laboratory), TMB or AEC substrate (if streptavidin-HRP is used) (*see* **Note 2**).

2.4 Plate Washer and ELISPOT Analyzer (Recommended)

1. Plate washer (e.g., BioTek Elx405 plate washer, as used in author's laboratory).
2. ELISPOT plate analyzer and corresponding software (e.g., CTL ImmunoSpot analyzer, as used in author's laboratory).

3 Methods

(*See* **Notes 3** and **4**)

3.1 Coating of Plates with Capture Antibody

1. Dilute capture mAb in sterile PBS at suggested or titrated optimal working concentration.
2. Coat the ELISPOT plate with diluted capture mAb at 100 μl/well; seal and store plate with lid in humid box at 4 °C, overnight.

3.2 Antigen Recall and Cell Culture (See Note 5)

1. Wash plate four times with sterile PBS at 200 μl/well.
2. Block plate with blocking buffer at 200 μl/well at room temperature, for 1 h.
3. Wash plate again three times with sterile PBS at 200 μl/well.
4. Add antigens (1–20 μg/ml or desired concentration for particular experiment) or positive control (e.g., PHA at 5 μg/ml; anti-CD3 mAb at 1 μg/ml) diluted in serum-free medium at 100 μl/well.
5. Resuspend cells harvested from particular tissue/organ (e.g., spleen, lymph nodes, or *see* **Notes 6** and **7**) at proper concentration with serum-free medium and add onto the plate at 100 μl/well using a pipettor with 200 μl large-orifice pipet tips (if two types of cells will be plated, e.g., when APCs are required, then add 50 μl/well with double the cell concentration for each type) (*see* **Notes 8** and **9**).
6. Tap sides of the plate to prevent cell clusters along the wall at the bottom of the wells.
7. Incubate the plate at 37 °C with 5.0 % CO_2 for 24 h (Th1 and Th17 cytokines) or for 48 h (Th2 cytokines and GM-CSF).

3.3 Cytokine Detection

1. Wash plate three times with PBS at 250 µl/well using plate washer (e.g., BioTek plate washer) and soak for approximately 30 s after each wash.
2. Wash plate four times with PBST at 250 µl/well using plate washer and soak for approximately 30 s after each wash.
3. Dilute detection mAb in PBST with 1 % BSA at suggested or titrated optimal working concentration.
4. Add diluted detection mAb at 100 µl/well to the plate; seal and store plate with lid in humid box at 4 °C, overnight.

3.4 Plate Development and Analysis (See Note 2)

1. Wash plate four times with PBST at 250 µl/well using plate washer and soak for approximately 30 s after each wash.
2. Dilute streptavidin alkaline phosphatase conjugate in PBST with 1 % BSA (1:2,000 or at optimal titrated concentration).
3. Add diluted streptavidin alkaline phosphatase at 100 µl/well to the plate and incubate at room temperature for 2 h.
4. Wash plate four times with PBS at 250 µl/well using plate washer and soak for approximately 30 s after each wash.
5. Develop the plate by adding BCIP/NBT phosphatase substrate at 200 µl/well.
6. After distinguishable and dark spots have formed, stop reaction by rinsing the wells gently with distilled water (*see* **Note 10**).
7. After air drying of the plate (usually after 1–2 h), scan and count the spots by ELISPOT plate analyzer by determining actual spot size and intensity parameters optimized over background (Fig. 1e). New generations of ELISPOT analyzers provide the option to analyze spots based on fluorescent labeled antibodies. Analysis of fluorescent spots has to be adjusted accordingly.

4 Notes

1. Listed are antibody pairs for cytokines commonly used in our laboratory. Different companies offer specific clones of monoclonal antibodies. However, the optimal working concentration for each antibody may vary because of differences between commercial antibody batches, even if they are obtained from the same company. When using a new batch of antibody, titration is recommended in order to determine optimal working concentrations, unless it is pre-titrated and to be used at a particular concentration per the instructions of a commercial ELISPOT kit. If other pairs of mAb clones are used, i.e., for cytokines not listed in this protocol, it is highly recommended to perform a careful cross-titration of both capture and

detection mAb to determine optimal working concentrations. To titrate antibody pairs for a cytokine of interest, usually 1–4 μg/ml of the capture mAb per well provides a good starting point. Similarly, a starting range of 0.25–2 μg/ml of detection mAb is recommended.

2. In the case of cytokine FluoroSpot, where fluorescent conjugate is used instead of enzyme conjugate, substrates will not be needed since there will not be a development step. After incubation with streptavidin-fluorochrome conjugate and washing steps, the plate will be air-dried and analyzed immediately (protected from light).
3. Procedures 3.1 and 3.2 should be performed with aseptic (sterile) technique in a biosafety cabinet. Procedures 3.3 and 3.4 can be performed on a regular laboratory bench.
4. Using ELISPOT for detecting specific antibody-secreting B cells, the same principles and steps can be followed as for cytokine ELISPOT assay with the exception that, instead of coating the ELISPOT plates with capture antibodies, the plates are coated with specific antigen (e.g., peptide or protein, 1–20 μg/ml).
5. The same principles for the ELISPOT assay apply when antigen-induced cytokine responses are not the objective, e.g., testing cytokine responses induced by mitogens, TLR ligands, or spontaneous cytokine responses by immune cells. However, the incubation period/conditions and mAb concentrations for the cells of interest on the ELISPOT plates have to be determined and optimized accordingly.
6. Isolation of lymphocytes/mononuclear cells from lymphoid tissues or peripheral blood: after crushing the tissue and obtaining the cell suspension, remove debris, such as connective tissues, by filtering through a cell strainer (e.g., 40 μm type). Remove red blood cells (RBCs) as needed (e.g., peripheral blood samples) using a Ficoll gradient (e.g., Lympholyte-M, Cedarlane laboratories, as used in author's laboratory).
7. Isolation of tissue/organ infiltrating lymphocytes (e.g., from brain, lung, liver, pancreas, and heart): perfusion of the animals with PBS or HBSS medium is recommended to avoid contamination with lymphocytes from peripheral blood. Tissue dissociation steps will be needed and vary depending on specific organs. For isolating mononuclear cells from the brain, the myelin removal process is recommended (e.g., myelin-removal beads, Miltenyi Biotec, as used in author's laboratory). For organs such as lung and heart, tissue dissociation and enzyme digestion steps are required (tissue-specific dissociation kit and tissue dissociator, Miltenyi Biotec). For the pancreas, enzyme digestion is required in order to separate infiltrating lymphocytes from pancreatic

tissues (Collagenase P, Roche Diagnostics, as used in author's laboratory). After tissue dissociation (e.g., liver, lung, or heart tissue), a Ficoll gradient (Lympholyte-M, Cedarlane laboratories) can be used to remove debris and RBCs in cell suspensions prior to ELISPOT.

8. The number of cells per well for the ELISPOT plate should be titrated and determined for optimal spot profile/separation for each particular type of tissue. For reference, the concentrations of cells most commonly used are listed below. In studies where purified T lymphocytes are used, or where the number of cells/well is less than 1×10^6 cells, it is recommended to provide additional feeder cells (e.g., spleen cells) or antigen presenting cells (APCs) during the activation period.

   Usual concentration of cells per well for selected tissues:

   Mouse draining lymph node cells: 0.5×10^6 cells/well.

   Mouse splenocytes: $0.5–1 \times 10^6$ cells/well.

   Mononuclear cells extracted from mouse brain (e.g., in experimental autoimmune encephalomyelitis, EAE studies): 20,000–50,000 cells/well.

   Mouse T cell lines: 500 or 1,000 cells/well.

   Mouse T cell clones: 250–1,000 cells/well.

   Mouse APCs: splenocytes or thymocytes, 0.5×10^6 cells/well; B-cells: 40,000 cells/well.

   Human PBMCs: $0.5–1 \times 10^6$ cells/well.

   Human T cell lines: 10,000–20,000 cells/well.

   Human APCs: autologous PBMCs at 0.5×10^6 cells/well.

9. It is important to accurately determine the live cell count for each sample. Our laboratory routinely determines live cells in single-cell suspension using acridine orange (AO) and ethidium bromide (EB) staining (working solution: mixture of 1 μg/ml of AO and 1 μg/ml of EB prepared from 1 mg/ml stock solution in PBS). The cell suspension is mixed with AO/EB working solution at 1:2–1:20 dilution (depending on the approximate concentration of cells) and counted with the fluorescent microscope using the FITC channel. Live cells will appear green, and dead or apoptotic cells will show as orange under the microscope.

10. The development time necessary to yield optimal spot morphology and intensity over the background varies for each cytokine and each particular sample. Therefore, the developing plate should be carefully monitored in order to avoid overdeveloping which otherwise may result in a high background and interfere with analysis by the ELISPOT analyzer.

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Isolation of Central Nervous System (CNS) Infiltrating Cells

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Abstract

Leukocyte infiltration of the central nervous system (CNS) occurs under certain pathogenic conditions and most often results in severe disorders. Therefore, the isolation and analysis of such infiltrating cell populations is necessary for elucidating the underlying pathogenic mechanisms. Here we describe a simple and straightforward protocol for cell isolation from the inflamed CNS, which combines mechanical dissociation and enzymatic degradation of the tissue. Additionally, purification by Percoll gradient centrifugation provides a great yield of the infiltrating material. The isolated cells can be further used for downstream applications such as cell sorting, cellular or molecular analysis.

Keywords: CNS, EAE, Multiple sclerosis, Percoll, T cells, Blood–brain barrier, Protocol

1 Introduction

The integrity of the blood–brain barrier (BBB) determines proper trafficking of nutrients and (cellular) mediators into and from the CNS parenchyma, and therefore represents a key feature of the healthy individual. However, under certain circumstances disruption of the BBB leads to severe dysfunctions. Thus, BBB breakdown has been associated, among others, with Alzheimer's disease, Parkinson's disease, brain infections, stroke, epilepsy, and multiple sclerosis (MS) (1). MS is a common neurodegenerative disorder that affects mainly Caucasians in high-income countries and the number of people suffering from MS has increased in the last years and reached about 2.5 millions all over the world (2). The disease progression is characterized by the presence of multiple inflammatory and demyelinating lesions within the brain and spinal cord, which result in axonal damage and consequently in clinical symptoms such as sensory and motor impairment, ataxia, spasticity, fatigue, and cognitive impairment (3). MS is believed to be of autoimmune nature and has a clear inflammatory component. Despite intensive investigations during past decades the exact mechanisms of disease onset and progression still remain unknown. To study MS in more detail an animal model termed experimental autoimmune encephalomyelitis (EAE) has been established. EAE mimics many of the MS features in rodents and, despite all its

limitations, remains the best tool to study the disease in vivo. Like in MS, EAE progression is also characterized by inflammation and accumulation of infiltrating peripheral cells within the CNS. Interestingly, EAE clinical symptoms can be different according to the main site of inflammation being the brain or spinal cord (4). Therefore, CNS infiltrating cell isolation is a crucial and indispensible step for understanding the mechanisms of MS/EAE pathogenesis.

Here we describe a detailed protocol for the isolation of CNS infiltrating cells from animals suffering from mouse models of the aforementioned disorders with CNS associated immune responses, such as EAE. An essential basis for the protocol is the cardiac perfusion of the animals with normal saline solution to avoid possible peripheral blood cell contamination of the CNS samples. In the second step a combination of mechanical dissociation and enzymatic degradation of the extracellular matrix proteins is implemented to achieve a single cell suspension. Further purification with the colloid Percoll is necessary to enrich for CNS infiltrating cells. Pecoll is not toxic to the cells under these conditions and does not interfere with many laboratory applications, such as electronic counting instruments or fluorescent activated cell sorting (FACS). Therefore, isolated CNS infiltrating cells may be used for further analysis and characterization.

Importantly, the protocol is not designed to isolate only mononuclear CNS infiltrating cells but rather attempts to purify these cells from debris, extracellular components, and most importantly the myelin. The critical step of a discontinuous three-layer Percoll gradient leads to enrichment of leukocytes as well as CNS resident cell types (such as microglia (5)) in the interphases. Beneficially, these CNS resident cell types may be used as internal control for infiltrating cell quantification. Therefore, additional purification procedures are required to isolate specific CNS infiltrating cell subsets.

Total numbers of CNS infiltrating cells are strongly correlated with EAE severity and the stage of disease progression. For example, to yield the highest amounts of T cells from the diseased CNS the isolation should be performed at the peak of disease with pooled brain and spinal cord material. Using the here described method, isolation of CNS (infiltrating) cells from mice with fully paralyzed hind limbs (score 3.5), partially (score 4) or fully (score 4.5) paralyzed front limbs routinely yields 1.5–3.5 million cells, including about 0.5 million CD90 positive T cells (Fig. 1a). Importantly, the isolated cells may be further used for downstream application, e.g. the flow cytometric analysis to distinguish infiltrated lymphocytes (CD45+CD11b−), macrophages ($CD45^{high}$CD11b+) and microglia ($CD45^{low}$CD11b+) (Fig. 1b). Moreover, different effector T cell subsets maybe be identified and characterized based on their characteristic cytokine expression (Fig. 1c).

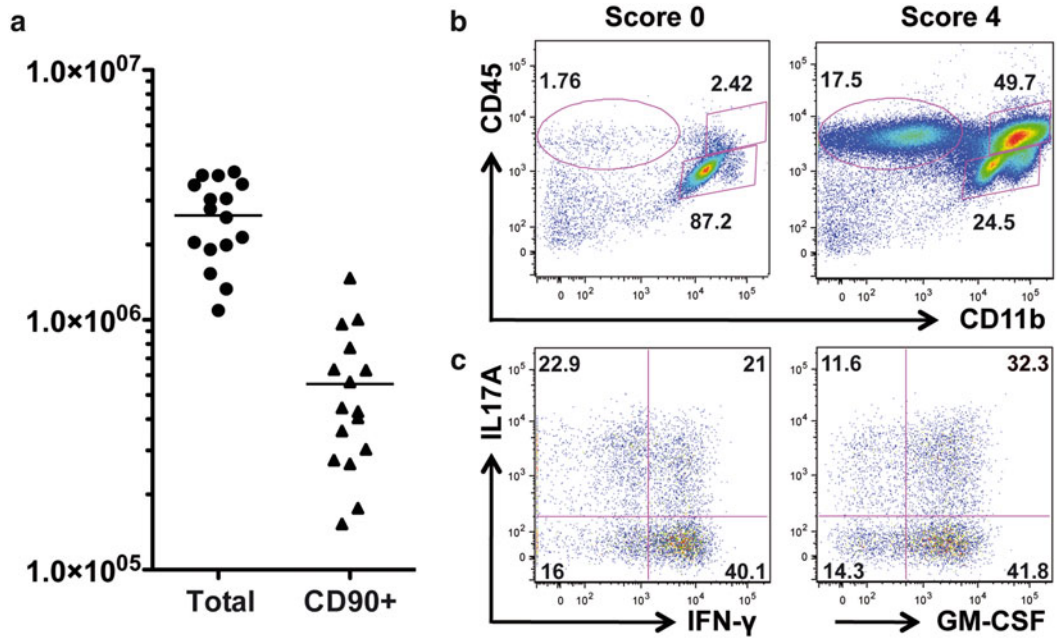

Fig. 1 Isolation of CNS infiltrating cells from C57Bl/6 mice with EAE. (**a**) Absolute numbers of live and CD90 positive cells. Data represents CNS isolated material from 19 EAE diseased mice, with clinical scores ranging from 3.5 to 4.5, from 7 independent experiments. (**b**) Surface markers expression profile of CNS infiltrating lymphocytes (CD45+CD11b−), macrophages and microglia represents greater amounts of inflammatory cells in EAE diseased mouse (score 4) compare to nondiseased mouse (score 0). (**c**) Cytokine expression profile of CNS infiltrated CD4+CD40L+ T cells isolated from a mouse with clinical score 4. Cells were stimulated ex vivo in the presence of MOG peptide and Brefeldin A for 6 h

2 Materials

Prepare all equipment and materials in advance, as the time is critical for cell survival during the isolation procedure. Only digestion solution and Percoll fractions should be prepared shortly before usage.

2.1 Equipment

1. Surgical instruments: scissors, forceps, i.v. perfusion system, razor blades.
2. Suppliers: 100 mm petri dishes, 15 ml Falcon tubes, 5 ml and 10 ml medical syringes, hypodermic needles (BD): 0.9 × 40 mm 20 G "yellow," 1.2 × 40 mm 18 G "red," 0.9 × 70 mm 20 G "long yellow" (*see* **Note 1**).
3. Equipment: vortexer, centrifuge with swing-out bucket rotor, water bath.

2.2 Reagents

1. Mouse anesthesia: Isofluran (*see* **Note 2**).
2. 70 % Ethanol.

3. Perfusion solution: Normal saline (0.9 % w/v NaCl) (*see* **Note 3**).
4. Phosphate buffered saline (PBS), without Calcium and Magnesium.
5. PBS, with Calcium and Magnesium.
6. 10×PBS, without Calcium and Magnesium.
7. Hanks' Balanced Salt Solution (HBSS), without Calcium and Magnesium (*see* **Note 4**).
8. Fetal calf serum (FCS).
9. Cell isolation/storage buffer (PBS/FCS): PBS supplemented with 2 % FCS.
10. Digestion solution: PBS with Calcium and Magnesium, 1.5 mg/ml Collagenase II, 50 μg/ml DNase I (*see* **Note 5**).
11. Stock Isotonic Percoll (SIP) solution: 90 % Percoll plus 10 % 10×PBS, without Calcium and Magnesium (*see* **Note 6**).
12. 30 %—Percoll fraction: 30 % SIP plus 70 % PBS, without Calcium and Magnesium.
13. 37 %—Percoll fraction: 37 % SIP plus 63 % HBSS, without Calcium and Magnesium.
14. 70 %—Percoll fraction: 70 % SIP plus 30 % PBS, without Calcium and Magnesium (*see* **Note 7**).

3 Methods

Keep biological material on ice unless otherwise specified. The stated volumes are calculated for the whole CNS, i.e. brain plus spinal cord of one mouse. Cell isolation from separated CNS parts is possible with reduced volumes. If further culturing of the cells is required, the isolation should be performed under sterile conditions, e.g. with sterile instruments, suppliers, solutions and under a laminar flow cabinet (*see* **Note 8**).

3.1 CNS Tissue Isolation and Dissociation

1. Deeply anesthetize mouse, make sure there is no pain reflex (*see* **Note 9**).
2. Spray mouse with ethanol, cut the skin, and open thoracic cavity. Make a small cut in the right atrium of the heart and insert the perfusion needle 0.9 × 40 mm into the left cardiac ventricle (*see* **Note 10**).
3. Run 40–50 ml of the perfusion solution for about 3–5 min through cannula with low drip until the effluent solution from the right atrium doesn't consist of blood, i.e. become fully transparent (*see* **Note 11**).

4. Decapitate mouse, cut the skin, and isolate the brain out of the skull into a petri dish. Cover isolated brain with 3 ml PBS/FCS (*see* **Note 12**).
5. From the mouse body, cut the back skin longwise from the cranial end until the tail basis. Cut the ribs and separate the spinal column down to lumbar region.
6. Fill up 10 ml syringe with 1.2 × 40 mm needle with PBS/FCS. Fix the spinal column with forceps and insert the needle into the spinal canal at the caudal end. Flush out the spinal cord with PBS/FCS into petri dish (*see* **Note 13**).
7. Combine spinal cord and brain in one petri dish, if cell isolation is desired from the whole CNS.
8. Cut CNS tissue into small pieces (about 1 mm^2) by using a razor blade and collect everything in a 15 ml Falcon tube.
9. Centrifuge at 300 × *g*, 5 min, 4 °C. Remove the supernatant.
10. Resuspend in 1 ml digestion solution by using vortexer and incubate for 30 min, 37 °C in water bath.
11. Stop the reaction by placing Falcon tube on ice and adding 3 ml PBS/FCS.
12. Homogenize digested tissue pieces by using the 5 ml syringe with 1.2 × 40 mm needle. Intensive collection and removal for about 3–5 times is necessary.
13. Add 8 ml PBS/FCS and centrifuge at 300 × *g*, 5 min, 4 °C. Remove the supernatant (*see* **Note 14**).
14. Resuspend the pellet in 4 ml 70 %—Percoll fraction by using vortexer and collect in 5 ml syringe with 0.9 × 70 mm needle.

3.2 CNS Infiltrating Cell Purification

1. Add 4 ml 30 %—Percoll fraction into a new 15 ml Falcon tube and carefully underlay with 4 ml 37 %—Percoll fraction by using 5 ml syringe with long 0.9 × 70 mm needle. Avoid air bubbles and mixing fractions due to turbulence (*see* **Note 15**).
2. Carefully underlay 4 ml of 70 %—Percoll fraction containing digested CNS into the Falcon tube from step 1 (already containing the other two Percoll fractions). Again, use the syringe with 0.9 × 70 mm needle like in step before. Avoid air bubbles and residual tissue clumps (*see* **Note 16**).
3. Perform Percoll gradient separation by centrifugation for 40 min at 500 × *g*, RT with the lowest acceleration settings and without brake. After centrifugation the tube should contain a viscose myelin-enriched layer on top. The CNS infiltrating cells and microglia should be visible as an opaque white ring at the interphase of middle 37 % (red) and lower 70 % (white) Percoll fractions.
4. Carefully remove the upper myelin-enriched layer (*see* **Note 17**).

5. Carefully harvest cells from the interphase described above and transfer into new 15 ml Falcon tube (*see* **Note 18**).
6. Add 10 ml PBS/FCS, vortex, and centrifuge at 300 × *g*, 5 min, 4 °C (*see* **Note 19**).
7. Resuspend cells in PBS/FCS and proceed for further analysis.

4 Notes

1. Using syringes with Luer lock are preferential for safety reasons.
2. Isofluran can be exchanged with other anesthesia. Follow the institutional biohazard regulations.
3. Other isotonic solutions can be used instead of NaCl, for example 1×PBS without Calcium and Magnesium.
4. Using HBSS with Phenol Red will help to better visualize different Percoll fractions.
5. Make aliquots of 2 mg/ml DNase I and store at −20 °C.
6. Concentration is represented here as volume/volume parts, i.e. take nine parts of Percoll and one part of 10×PBS to prepare SIP.
7. Brief calculation for infiltrating cell isolation from 1 mouse CNS (brain plus spinal cord):

   5.5 ml SIP = 4.95 ml Percoll + 0.55 ml 10×PBS

   4 ml 30 %—Percoll = 1.20 ml SIP + 2.80 ml 1×PBS

   4 ml 37 %—Percoll = 1.48 ml SIP + 2.52 ml 1×HBSS

   4 ml 70 %—Percoll = 2.80 ml SIP + 1.20 ml 1×PBS

   Prepare different Percoll gradient fractions shortly before usage. Cold Percoll could result in forming cell aggregates; therefore, solutions should be at RT.
8. Working in a team, especially when handling large sample numbers, significantly decreases the time of cell isolation, which is crucial for yield and quality.
9. To check pain reflex pinch the skin with the forceps. If there is a reaction perform longer anesthesia. Important! Deep anesthesia should still keep the heart beating, which is necessary for successful cardiac perfusion. Cervical dislocation is not acceptable as it may disrupt blood vessels and disturb CNS perfusion.
10. The notched right atrium will allow perfusion solution to go out of circulation.
11. Alternatively, instead of i.v. perfusion system, a perfusion pump or manual syringe perfusion can be used. Follow the color change of the liver from dark red to light brown as indicator of successful perfusion.

12. A fully white brain with nonvisible blood vessels is a good indication of successful perfusion. Avoid any residual blood spillover from surrounding tissues. If such blood contamination occurred, wash CNS tissue with high-volume PBS/FCS. In case some parts of the brain are desired for further histological analysis be careful with the cutting and removal of cranium bones.
13. The needle diameter is just slightly below the internal spinal canal diameter. Therefore, tight junction should prevent retrograde spinal cord flushing. If the resistance during flushing is too strong make sure the spinal column is straightened. Once the spinal cord is flushed out, repeat the procedure, now from the cranial end. Avoid any residual blood contamination from surrounded tissues. If blood contamination occurs, wash with high-volume PBS/FCS.
14. In that stage the pellet is loose, be careful with supernatant removal.
15. Filling up the syringe with 5 ml 37 %—Percoll solution and release only 4 ml will help to avoid air bubbles. A clear separation line should be visible at the 30–37 % junction.
16. Be careful when releasing the residual volume from the syringe, this is the most critical step in air bubble generation.
17. Also remove the white ring on top of red fraction if that have had appeared.
18. Collecting cells with 1 ml pipet tips is useful for this purpose. Usually 2 ml is enough for proper cell collection.
19. Proper mixing is necessary for washing out the residual Percoll solution.

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Methods for Ex Vivo Analysis of Immune Cell Function from the Central Nervous System

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Abstract

Experimental autoimmune encephalomyelitis (EAE) is an animal model commonly used to investigate the inflammatory response in organ-specific autoimmunity and a model of the early immune responses of multiple sclerosis.

This protocol outlines the methods used for the processing of peripheral immune tissues, the spleen and draining lymph nodes, as well as the site of inflammation, the central nervous system (CNS), for analyzing immune cell phenotype and function during murine EAE.

Keywords: Experimental autoimmune encephalomyelitis, CNS, Autoimmune disease, Inflammatory cells, Cytokines, Transcription factors, T cells

1 Introduction

EAE is a disease model that is widely used to study autoimmune inflammation within the CNS and the role that immune cells play in the induction and subsequent resolution of CNS inflammation. The model can be induced via two methods: active EAE, where the induction of disease relies on immunization with myelin antigens in adjuvant, or passive EAE induced by the transfer of polarized myelin-reactive CD4+ T cells without any requirement for immunization of the host mice. In both cases the myelin-reactive CD4+ T cells migrate into the CNS provoking immune-mediated damage to the myelin sheath. Various transgenic mouse lines have been used to elucidate the role of individual cells and genes in the progression and resolution of CNS inflammation in EAE.

To understand the complex relationships between the various cells and the specific action of certain genes involved during the various stages of EAE, it is important to have a reliable method to obtain the cells involved in the inflammatory response and to be able to compare the function of these retrieved cells. Obtaining cells of interest from the lymphoid tissues is relatively straightforward and quick. Active EAE allows investigation of both the spleen

and the lymph node draining the site of immunization, due to the establishment of an antigen depot within the periphery of mice, whereas passive EAE is best investigated using the spleen.

The removal and processing of the CNS, which has a very different composition and relatively few immune cells involved in the inflammatory response, is more involved and time consuming. CNS tissue must be mechanically disrupted and structural proteins degraded by enzyme action in order to obtain mononuclear cells. Cells must also be separated away from the large amount of fat that is present within the CNS which can interfere with downstream processing. Here we outline the method for the isolation of mononuclear cell suspensions and the downstream analysis of these cells to determine cellular phenotype and function.

2 Materials

2.1 Isolation of Mononuclear Cells from Peripheral Lymphoid Organs and CNS

1. Wash medium: RPMI-1640.
2. RPMI-10 %: RPMI-1640 supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol.
3. Sterile gauze 150 μm pore.
4. 15 ml Falcon conical centrifuge tubes.
5. RBC lysis buffer: Containing 8.3 g/l ammonium chloride in 0.01 M Tris–HCl.
6. Sterile PBS (without Mg^{2+} and Ca^{2+}).
7. Trypan blue.
8. Percoll gradients: 30 and 70 % Percoll (density of 1.135 g/ml) solutions diluted with wash medium.
9. CNS digestion buffer: 2.5 mg/ml Collagenase IV (Lorne Laboratories, UK) + 1 mg/ml deoxyribonuclease (DNase) diluted in wash medium.
10. 18 G Blunt fill needle 1.2 mm × 40 mm.
11. 25 G needle.
12. 7 ml flat-bottom plastic bijou.
13. Hemocytometer.

2.2 Phenotypic Analysis of Mononuclear Cells

1. Ex vivo medium: X-VIVO-15 serum-free medium with gentamycin supplemented with 2 mM L-glutamine and 50 μM 2-mercaptoethanol.
2. 24-, 48-, and 96-well flat-bottom cell culture plates.
3. Myelin-derived peptides: For example MOG(35–55) (MEVGWYRSPFSRVVHLYRNNGK), or MBP Ac1-9

(Ac-ASQKRPSQR) (Cambridge Research Biochemicals, Teesside, UK).

4. FACS buffer: PBS supplemented with 2 % heat-inactivated FCS and 0.1 % sodium azide.
5. PMA: Phorbol 12-myristate 13-acetate 100 μg/ml prepared in DMSO.
6. Ionomycin: 1 mg/ml stock prepared in DMSO.
7. Golgi inhibitor: Use as per the manufacturer's directions (**Note 1**).
8. FACS tubes: 5 ml 12 × 75 mm round-bottom tubes.
9. Fixable live dead stain: Fixable viability dye eFluor® 455UV (eBioscience). Use as per the manufacturer's instructions (**Note 2**).
10. 1 % PFA: 1 % paraformaldehyde in PBS.
11. BD fixation buffer: BD Cytofix/Cytoperm.
12. BD permeabilization buffer: BD 10× Perm/Wash diluted as per the manufacturer's instructions.
13. eBioscience fixation buffer: eBioscience FoxP3 fixation/permeabilization buffers made up as per the manufacturer's directions.
14. ^{3}[H]-thymidine: Methyl ^{3}[H] thymidine (**Note 3**).
15. Recommended antibodies and stains for flow cytometry: CD4 Brilliant Violet 650™ (clone RM4-5, Biolegend), CD11b APC-efluor780™ (clone M1/70, eBioscience), Foxp3 eFluor 450™ (clone FGK-16s, eBioscience), Ly6c FITC (clone AL-21, BD Bioscience), Ly6G Alexa Fluor™ 700 (clone 1A8, BD Bioscience), MHC class II (clone relevant to genetic background, i.e., M5/114.15.2, eBioscience) (**Note 3**).
16. Proprietary ELISA kits for detection of mouse IFNγ, IL-17A, GM-CSF, TNFα, or IL-10, for example Ready-SET-Go! (eBioscience).

3 Methods

Perform all procedures using good sterile technique and a class 2 biological safety cabinet (**Note 4**).

3.1 Isolation of Mononuclear Cells

This section outlines the isolation of the mononuclear cells from the spleen, lymph node (draining the site of inflammation in active EAE induction), and CNS. Following isolation, cells can be used for downstream analysis to determine cell phenotype and function. Using this technique, isolated cell populations of interest can be further purified by fluorescence-activated cell sorting (FACS) for molecular studies such as real-time PCR.

3.1.1 Preparation of Single-Cell Suspension from Mouse Spleens and Draining Lymph Nodes

1. Harvest the draining lymph nodes (dependent on immunization site) and spleen as required into wash medium on ice or at 4 °C until tissue can be processed.
2. Create a single-cell suspension by pressing cells through 150 μm sterile gauze or equivalent cell strainer in wash medium to a 10 ml final volume.
3. To wash/pellet cells, centrifuge at 300 × *g* for 5 min and discard supernatant (as for all subsequent washes unless stated).
4. Lyse red blood cells in spleen samples at room temperature for 2 min using 2 ml RBC lysis buffer per spleen. The reaction is stopped by the addition of 10 ml RPMI-10 %. Pellet cells, resuspend in wash buffer, and count viable cells using trypan blue exclusion.

3.1.2 Preparation of Mononuclear Cell Suspension from the CNS (Adapted from (1))

1. Euthanize mice by CO_2 asphyxiation.
2. Perfuse the mice with 10 ml ice-cold PBS transcardially via the left ventricle using a 25 G needle.
3. Remove the skin covering the skull and spinal column.
4. Make three cuts, between the eyes, below the base of the skull and at the base of the spine just above the pelvis.
5. The spinal cord can be extracted by use of a blunt needle and hydrostatic pressure. Dissect out the entire spinal column, and fill a 2 ml syringe with cold wash medium with blunt needle attached. Insert the needle into the base of the spinal column and gently insert the needle. Force the spinal cord out by applying pressure to the syringe and transfer to a 7 ml plastic bijou (**Note 5**).
6. The brain can be removed whole by incision of the skull midline and carefully pulling the skull apart. Place into the same tube as the spinal cord.
7. Remove the wash medium from tube containing the tissue and add 400 μl CNS digestion buffer.
8. Cut the tissue into small pieces using scissors and then manually disaggregate using a 1 ml syringe until complete disruption is achieved.
9. Incubate in a water bath at 37 °C for 40 min.
10. Transfer the sample to a 15 ml Falcon, rinse the bijou with wash medium, and add to the 15 ml tube.
11. Add 10 ml wash medium and pellet the cells by centrifugation at 300 × *g* without a brake for 5 min at room temperature.
12. Carefully discard the supernatant so as not to disrupt the soft pellet.
13. Resuspend the pellet in 5 ml 30 % Percoll.

14. Underlay 5 ml 70 % Percoll using a pipette to get a clean discontinuous gradient interface (**Note 6**).
15. Layer 2 ml of wash medium on top of the 30 % Percoll to help with removal of fatty layer following centrifugation.
16. Centrifuge for 20 min at 530 × *g* without a brake.
17. Remove and discard the layer of fat at the top of the tube (**Note 7**).
18. Using a pipette collect cells from the 70:30 interface and transfer to a clean 15 ml Falcon tube.
19. Add 10 ml wash medium, centrifuge at 300 × *g* for 5 min, and discard the supernatant (**Note 8**).
20. Resuspend the cell pellet in 1 ml RPMI-10 % and count viable cells by trypan blue exclusion.

3.2 Phenotypic and Functional Analysis of Inflammatory Cells

This section outlines the procedure for phenotypic and functional analysis of the cells once isolated from the peripheral organs or CNS. Figure 1 illustrates FACS analysis of T cell and monocyte fractions within the CNS infiltrate at the peak of disease. CD4+ T cells can be clearly visualized by counterstaining with CD11b to highlight antigen-presenting cell populations (monocytes, macrophages, and microglia) (Fig. 1a and **Note 9**). Once gated on the live, CD4+ CD11b− fraction, it is possible to determine the expression of cytokines and transcription factors within this population. Figure 2 illustrates intracellular cytokine staining of cells reactivated in vitro with their peptide of interest. Pro- and anti-inflammatory cytokines (IFNγ, IL-17, TNFα, GM-CSF, IL-10) can also be detected within the supernatants of cells subjected to recall assays using commercially available ELISA kits.

A major problem which challenges studies on monocytes/macrophages and their role in CNS inflammation is the ability to distinguish recruited cells from resident microglia in the CNS as both cell types have similar surface expression of the commonly used markers CD11b and CD45. Ly6c is highly expressed on infiltrating CCR2+ monocytes required for disease progression (2) and more recently has been used to define infiltrating monocytes from resident microglia which are Ly6c− (3, 4). Using the gating strategy outlined here in Fig. 1, retrieved cells are gated on live leukocytes and the CD11b+ cells identified (Fig. 1b). These CD11b+ cells can be subdivided into Ly6g+ (clone 1A8) neutrophils, Ly6c+ infiltrating monocytes, and Ly6c− microglia (Fig. 1c). Both Ly6c+ and Ly6c− populations show similar CD45 expression profiles (Fig. 1d). Having defined the infiltrating monocytes as CD11b+ CD45+ Ly6g− Ly6c+ cells, it is possible to look for further immune activation markers based on expression of MHC class II (Fig. 1e) and costimulatory molecules CD80, CD86, and CD40 (Fig. 1f–h). There has been much debate as to whether the

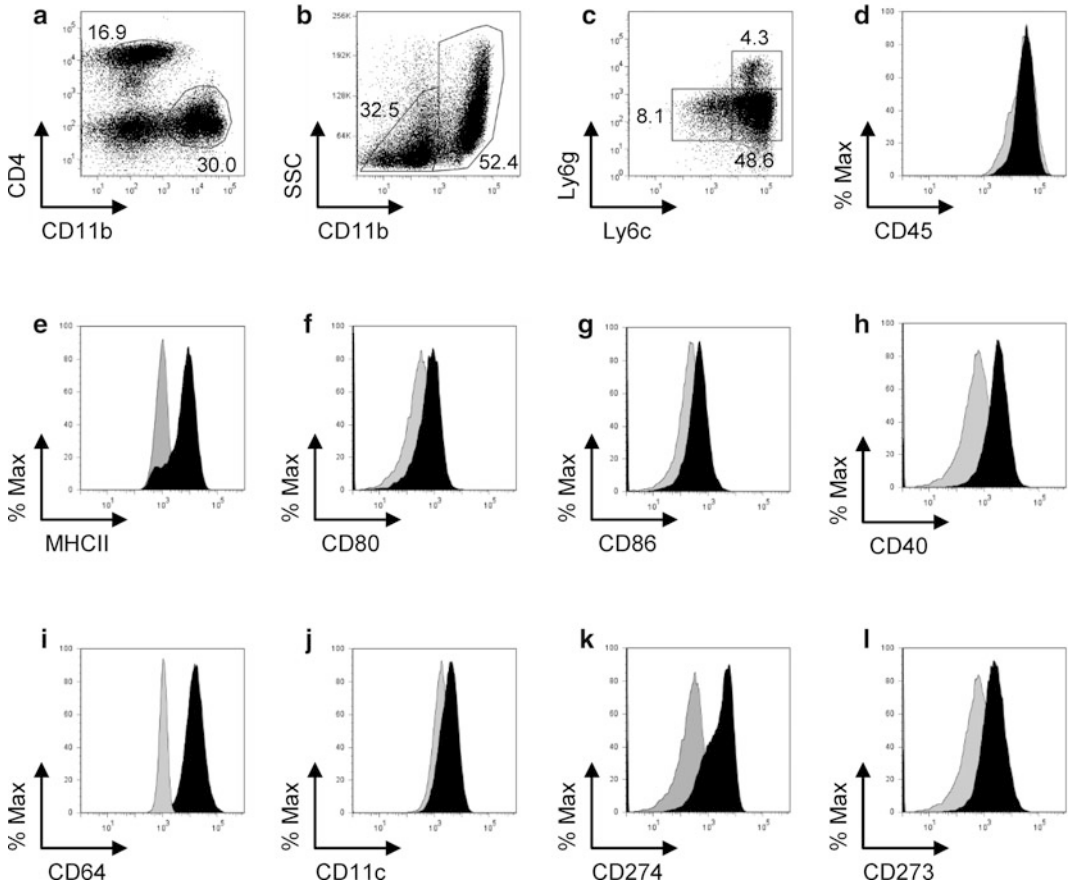

Fig. 1 Flow cytometric staining of the inflammatory infiltrate at the peak of EAE. (**a**) CD4 versus CD11b staining of CNS cells (initially gated using a live leukocyte gate). (**b**) CD11b+ cells, gated on live leukocyte gate. (**c**) Expression of Ly6c (monocytes) and Ly6g (neutrophils) within the CD11b+ gate. (**d**) Expression of CD45 on the Ly6c+ (*black*) or Ly6c− (*grey*) cells. (**e–l**) Ly6c+ monocytes in the CNS express high levels of MHC class II, CD64, intermediate expression of CD11c, the costimulatory molecules CD40, CD80, and CD86, and the two ligands for PD-1, CD274 (PD-L1), and CD273 (PD-L2). In histograms (**e–l**), *grey* = isotype control, *black* = specific antibody

infiltrating monocytes are able to differentiate into either macrophages or dendritic cells at the site of inflammation. In our hands, infiltrating monocytes have high expression of CD64 (Fig. 1l), which is selectively expressed on macrophages rather than dendritic cells (5) and show intermediate expression of CD11c (Fig. 1j) (6). This fits with the ability of monocytes to upregulate CD11c expression at sites of inflammation (7, 8). These Ly6c+ infiltrating monocytes have high expression of both CD273 (PD-L2) and CD274 (PD-L1) important for T cell regulation during inflammation (Fig. 1k–l) (9).

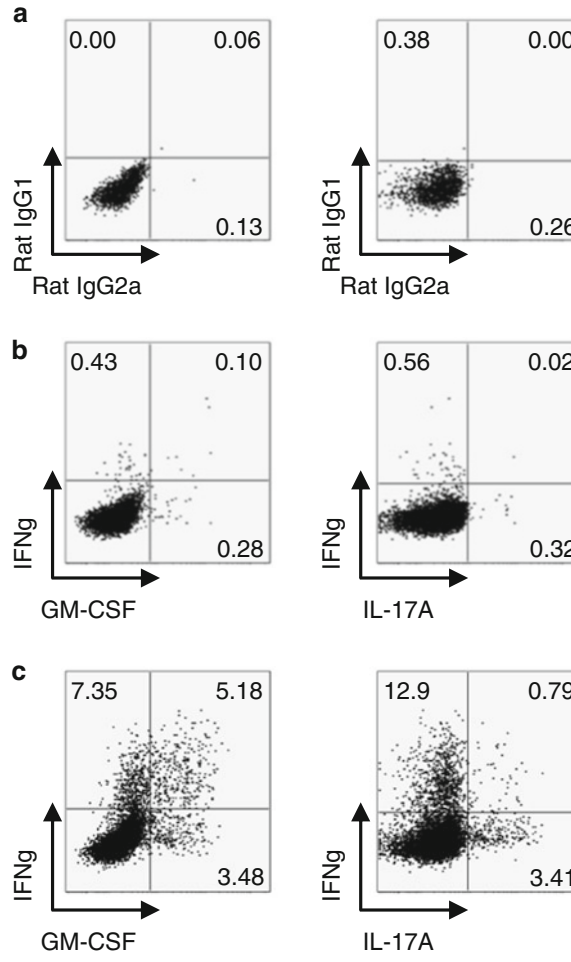

Fig. 2 Intracellular cytokine staining of CD4+ T cell population retrieved from the CNS at the peak of clinical EAE. CNS mononuclear cells were stimulated overnight +/− antigen prior to staining. (**a**) Isotype control staining. (**b**) Unstimulated cells. (**c**) MOG(35–55)-stimulated cells. Plots are gated on $CD4^{+}CD11b^{-}$ cells

3.2.1 Ex Vivo Surface Staining of Isolated Cells for Flow Cytometric Analysis

1. Transfer samples to FACS tubes and pellet the cells by centrifugation at 300 × *g* for 5 min. Discard the supernatant.
2. Add 2 ml PBS to the cell pellet and centrifuge at 300 × *g* for 5 min at 4 °C. Repeat this wash step to remove free protein.
3. Stain cells with fixable viability dye in PBS according to the manufacturer's specifications (**Notes 2, 10**).
4. Add 2 ml FACS buffer and centrifuge the cells at 300 × *g* for 5 min at 4 °C, and repeat.
5. Resuspend the cell pellet in 50 µl FACS buffer containing fluorescently conjugated antibodies for cell surface markers of interest and incubate at 4 °C for 30 min.

6. Repeat previous wash step 4.
7. Resuspend cells in 500 µl 1 % PFA or FACS buffer and store at 4 °C prior to acquisition.

3.2.2 Ex Vivo Transcription Factor Staining of Mononuclear Cells for Flow Cytometry

Once cells are stained for viability and surface molecules (Section 3.2.1), it is necessary to fix and permeabilize the cells prior to staining for transcription factors such as T-bet, RORγt, or Foxp3.

1. Add 500 µl eBioscience fixation buffer overnight at 4 °C.
2. Add 2 ml FACS buffer to each tube and centrifuge at 300 × *g* for 5 min at 4 °C. Discard the supernatant.
3. Add 50–100 µl 1× eBioscience permeabilization buffer containing relevant antibodies for 30 min at room temperature.
4. Wash and pellet cells twice in 2 ml FACS buffer.
5. Resuspend cells in 500 µl 1 % PFA or FACS buffer and store at 4 °C until acquisition.

3.2.3 Intracellular Detection of Cytokines in CD4+ T Cells Following Restimulation with Autoantigenic Peptide

1. Resuspend CNS cells at 4 × 10^6/ml, LN cells at 6 × 10^6/ml, and splenocytes at 8 × 10^6/ml, in either ex vivo medium for active EAE samples or RPMI-10 % for samples from passive EAE.
2. Add cells into an appropriately sized plate: 100 µl of CNS cells to a flat-bottom 96-well tissue culture plate, 500 µl LN cells to a 48-well culture plate or 1 ml to a 24-well culture plate, and 1 ml of splenocytes to a 24-well culture plate.
3. Simulate cells overnight with/without peptide appropriate to the EAE model used (final concentration of 20 µg/ml $MOG_{35–55}$ or 20 µg/ml $MBP_{Ac1–9}$) or for 4 h with 50 ng/ml PMA and 1 µg/ml ionomycin.
4. Culture cells at 37 °C, 5 % CO_2 in a humidified atmosphere.
5. For the final 3–5 h of culture add Golgi inhibitor (**Note 1**).
6. Resuspend cells, transfer to a FACS tube, and centrifuge at 300 × *g* for 5 min at 4 °C.
7. Fixable viability dye and surface molecule staining can be carried out as per Section 3.2.1, steps 2–6.
8. Resuspend the cell pellet in 500 µl BD fixation buffer and incubate for 20 min at 4 °C.
9. Wash cells in 1 ml BD permeabilization buffer and centrifuge at 300 × *g* from 5 min and repeat the wash.
10. Resuspend the cell pellet in 50–100 µl BD permeabilization buffer containing fluorescently conjugated antibodies for cytokines of interest and incubate at room temperature for 30 min.
11. Wash cells in 1 ml BD permeabilization buffer and centrifuge at 300 × *g* for 5 min and repeat the wash.
12. Resuspend cells in 300 µl FACS buffer and store at 4 °C until acquisition (**Note 11**).

3.2.4 Analysis of Secreted Cytokines Following Restimulation with Autoantigenic Peptide

1. Resuspend LN cells at 6×10^6/ml and splenocytes at 8×10^6/ml in ex vivo medium for ex vivo medium for active EAE samples or RPMI-10 % for samples from passive EAE.
2. Set up a tripling serial dilution of 100 μl peptide appropriate to the model (top concentrations, 60 μM $pMOG_{35–55}$, 200 μM $Ac_{1–9}$) in a 96-well flat-bottom cell culture plate and a row with no peptide.
3. Add 100 μl of each cell sample in per peptide concentration. Number of replicates is to be determined by the investigator.
4. Culture plates at 37 °C, 5 % CO_2 in humidified atmosphere, for 48 h for measuring IL-2, and 72 h for measuring other cytokines including IFNy, GM-CSF, IL-17, IL-10, and TNFα. Proliferation can be measured by 0.5 μCi ^{3}[H]-thymidine addition for the last 16–18 h of a 72-h culture, prior to harvesting and liquid scintillation counting.
5. Plates are stored at −20 °C prior to further analysis by ELISA or cytokine bead assay.

4 Notes

1. Alternative Golgi inhibitors are available, namely Brefeldin A and Monensin, which have different modes of action. These can be used separately or together, depending upon the cytokine of interest.
2. Optimization may be required. Antibodies and fixable live dead markers are available with many more different conjugates than those recommended here.
3. Methyl (^{3}H) thymidine is a radioactive material. Handling of radioactive material requires appropriate training and the complying of the code of practice for open-source radioactive material.
4. If cells are not required for culture, work can be carried out on the bench.
5. The spinal cord may catch as it is forced out; use blunt-ended forceps to gently retrieve the remaining tissue from the spinal column.
6. This is best achieved by having a handheld electronic pipette controller set to low or gravity setting so that the Percoll is added slowly and smoothly keeping the tip of the pipette just below the level of interface so that when the pipette is removed the interface is not disturbed.
7. Following centrifugation, be careful not to disrupt the different layers of the gradient. With a clean separation, a narrow band of cells should be seen at the 70:30 interface.

8. Depending on perfusion efficiency some red blood cells may still be present; these can be lysed as per Section 3.1.1, step 4.
9. CD4+ cells can be defined purely on CD4 expression. However we find that counterstaining with CD11b identifies auto-fluorescent APCs. Alternatively, co-staining with CD3 can be used.
10. Following the addition of light-sensitive fluorophores, steps should be taken to limit exposure to bright light.
11. Fixation of ICS staining in 1 % PFA can result in the bleaching of sensitive fluorochromes and the loss of signal.

Acknowledgements

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Part III

Biomarker Techniques

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Neurofilament Light Chain Determination from Peripheral Blood Samples

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Abstract

The loss of neurological function is closely related to axonal damage. Neurofilament subunits are concentrated in neurons and axons and have emerged as promising biomarkers for neurodegeneration. Electrochemiluminescence (ECL) based assays are known to be of superior sensitivity and require less sample volume than conventional ELISAs. Here, we describe a highly sensitive ECL based immunoassay for quantification of neurofilament light chain (NfL) in blood and CSF.

Keywords: Axonal damage, Neurofilament light chain (NfL), Biomarker, Electrochemiluminescence, Cerebrospinal fluid (CSF), Serum

1 Introduction

Highly sensitive methods for detecting soluble biomarkers for neuro-axonal damage are needed in neurodegenerative diseases.

Neurofilaments (Nf) are highly specific major structural proteins of neurons, consisting predominantly of four subunits: Nf light (NfL), Nf medium (NfM), and Nf heavy (NfH) chain and alpha-internexin (1). Nf are released in significant quantity following axonal damage or neuronal degeneration. Disruption to the axonal membrane releases Nf into the interstitial fluid and eventually into cerebrospinal fluid (CSF) and blood (*see* **Note 1**). Therefore, blood Nf levels could be useful for both predicting and monitoring disease progression and for assessing the efficacy and/or toxicity of future neuroprotective treatment strategies. Several previous studies have demonstrated the presence of NfH and NfL in CSF, which has been assumed to reflect brain pathology more accurately than the peripheral blood compartment (2–12). However, obtaining longitudinal CSF samples is relatively invasive, precluding the broader clinical use of Nf. In contrast to CSF, serial blood samples can readily be collected, and hence, reliable quantification of NfL in blood would be a major stride towards a biomarker of the course of neurodegeneration.

A commercially available ELISA (UmanDiagnostics NF-light® assay) uses two highly specific, non-competing monoclonal antibodies (47:3 and 2:1) to quantify soluble NfL in CSF samples but it cannot in its present form be used for analysis of blood samples (13).

Electrochemiluminescence (ECL) based assays are known to be highly sensitive, exhibit a broad dynamic range, and require small sample volume; ECL technology has demonstrated the ability to quantitate levels of nucleic acids, recombinant proteins, and bacterial and viral components in the sub-picogram range with increased precision compared to conventional ELISA (14–19).

Here we present a sensitive ECL-based NfL assay suitable for the quantification of NfL in serum at concentrations relevant to clinical settings.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C). Prepare and store all reagents at room temperature (unless indicated otherwise).

1. Capture monoclonal antibody (mAB) 47:3 (*see* **Notes 2** and **3**) (UmanDiagnostics, Umea, Sweden)
2. Biotinylated detector mAB 2:1 (10, 13) (*see* **Notes 2** and **3**) (UmanDiagnostics, Umea, Sweden)
3. MSD SULFO-TAG™ labelled streptavidin (MSD, Gaithersburg, MD; store at +4 °C) (Fig. 1).
4. Bovine serum albumin (BSA)

Fig. 1 Schematic illustration of the electrochemiluminescence NfL immunoassay. The carbon ink electrodes are coated with the capture mAB 47:3. The biotinylated detection mAB 2:1 binds to SULFO-TAG labelled streptavidin, which generates electrochemiluminescence signal

5. Ethylenediaminetetraacetic disodium salt (EDTA)
6. NaCl
7. 1× Phosphate buffered saline (PBS), pH 7.5
8. Tris base
9. Tween 20
10. 96-well plate including integrated screen-printed carbon ink electrodes on the bottom of the wells (96-well SECTOR standard plates, Meso Scale Discovery, Gaithersburg, MD).

2.1 Buffers

1. 10× Tris-buffered saline (TBS, 100 mM tris base and 1.5 M NaCl: 87.66 g NaCl and 12.1 g tris base per liter), adjusted to pH 7.5, using 10 N HCL as a stock solution.

On day 1 of the experiment the following buffers are prepared (amounts for one 96-well plate):

2. Working strength TBS (50 ml 10× TBS and 450 ml H_2O)
3. Blocking buffer: 3 % BSA in TBS (375 mg BSA in 12.5 ml TBS)
4. Wash buffer: 0.1 % Tween 20 in TBS (487.5 µl Tween 20 in 487.5 ml TBS)
5. Sample/standard diluent: 1 % BSA, 0.1 % Tween 20 in TBS (150 mg BSA in 15 ml of wash buffer)
6. Read buffer T (4×): 15 ml read buffer (MSD) in 15 ml H_2O

2.2 Standards

Bovine lyophilized NfL is obtained from UmanDiagnostics. Calibrators were serially diluted in standard diluent (10,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8125 pg/ml, and blank containing only diluent) (*see* **Notes 4** and **5**).

3 Methods

3.1 Coating of the Plate (Day 1)

1. Dilute mAB 47:3 (stock concentration: 1.1 mg/ml; working concentration: 1.25 µg/ml: 3,496 µl of PBS and 4 µl of mAB 47:3) in PBS.
2. Add 30 µl per well (*see* **Note 6**).
3. Seal wells with adhesive plate seals.
4. Incubate overnight at 4 °C.

3.2 Blocking of Unspecific Binding Sites (Day 2)

All the following incubation steps are done on a plate shaker (800 rpm, adhesive plate seals required for each incubation).

1. Rinse plate three times with 200 µl of wash buffer per well (*see* **Note 7**). Tap plate dry against absorbent paper.
2. Add 100 µl of blocking buffer per well (*see* **Note 8**).
3. Incubate plate for 1 h (*see* **Note 9**).

3.3 Adding Calibrators and Samples

1. Rinse plate three times with 200 µl of wash buffer per well. Tap plate dry against absorbent paper (*see* **Note 10**).
2. Add 25 µl of sample diluent to each well.
3. Add 25 µl of standard or sample in duplicate (*see* **Note 11**).
4. Incubate plate for 2 h.

3.4 Adding Detection Antibody (mAB 2:1)

1. Dilute mAB 2:1 (stock concentration: 0.58 mg/ml, working concentration: 0.5 µg/ml: 2,997.41 µl of sample/standard diluent and 2.59 µl of mAB 2:1).
2. Rinse plate three times with 200 µl of wash buffer per well. Tap plate dry against absorbent paper.
3. Add 25 µl of mAB 2:1 to each well.
4. Incubate plate for 1 h

3.5 Adding MSD SULFO-TAG™ Labelled Streptavidin

1. Dilute MSD SULFO-TAG™ labelled streptavidin (stock concentration: 0.5 mg/ml, working concentration: 0.25 µg/ml: 5,997 µl of sample/standard diluent and 3 µl of MSD SULFO-TAG™).
2. Rinse plate three times with 200 µl of wash buffer per well. Tap plate dry against absorbent paper.
3. Add 25 µl of MSD SULFO-TAG™ per well.
4. Incubate plate for 1 h.

3.6 Adding Read Buffer

1. Dilute read buffer T 4× 1:2 (10 ml H_2O and 10 ml read buffer T).
2. Rinse plate three times with 200 µl of wash buffer per well. Tap plate dry against absorbent paper.
3. Add 150 µl of read buffer (2×) per well (*see* **Notes 4**, **5** and **12**).

3.7 Detection of ECL Signal

1. The ECL signal is detected by photodetectors (*see* **Note 13**) (MSD SECTOR S 600, SECTOR Imager 2400 or QuickPlex SQ 120, see: http://www.mesoscale.com/CatalogSystemWeb/WebRoot/products/comparison.aspx for comparison of different hardware). A four-parameter weighted logistic fit curve is generated and sample concentrations extrapolated (*see* **Note 14**).

4 Notes

1. The assay is analytically and clinically validated for CSF and serum (20). Matched serum and plasma NfL levels from healthy controls showed high correlation ($n = 25$, Spearman's rho = 0.93, $p < 0.0001$) and strong agreement using Bland-Altman method comparison (bias: 3.92; Serum-plasma; 95 %

confidence interval: [−2.41, 10.25]; 95 % limits of agreement: [−26.15, 33.99]; Kuhle et al., unpublished data).

2. Store the monoclonal antibodies aliquoted at −20 °C.
3. The antibodies cross-react with NfL from mouse, rat, and bovine sources and can be used for research on these species.
4. Store calibrators and samples in polypropylene microcentrifuge tubes (70 µl each). We store batch-prepared, ready-to-use calibrators at −80 °C. Vortex before extracting calibrators/samples. Run calibrators on each plate.
5. In general the 10,000 and 2,000 pg/ml calibrators are only needed for CSF measurements.
6. When coating the plate make sure the entire surface of the carbon electrode is covered by fluid by gently tapping and tilting the plate.
7. We suggest an automated plate washer (e.g., Elx50 from BioTek), but washing with a multichannel pipette could also be done.
8. Avoid bubbles in wells at all pipetting steps (by reverse pipetting or pipetting to the bottom corner of the empty wells). Bubbles may lead to variation in results; bubbles introduced when adding read buffer may interfere with signal detection.
9. During the incubation steps, plates do not need to be shielded from light except direct sunlight.
10. Avoid letting the plate dry out at all stages.
11. We run at least three control samples on every plate to assess inter-assay variability (low, medium, and high concentration).
12. Keep time intervals consistent and short between adding read buffer and reading the plate (no shaking is necessary after adding read buffer).
13. Remove plate seals prior to reading the plate.
14. The assay has a wide dynamic range (linearity and parallelism have been validated up to 50 ng/ml) that allows accurate quantification of samples without the need for multiple dilutions.

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Detection of Autoantibodies Against Myelin Oligodendrocyte Glycoprotein in Multiple Sclerosis and Related Diseases

Melania Spadaro and Edgar Meinl

Abstract

Autoantibodies against myelin oligodendrocyte glycoprotein (MOG) occur in a proportion of patients with different inflammatory demyelinating diseases of the central nervous system, such as childhood multiple sclerosis (MS), acute disseminated encephalomyelitis (ADEM), and neuromyelitis optica spectrum disorders (NMOSD). We describe here in detail a sensitive cell-based assay that allows the identification of autoantibodies against MOG in serum.

Keywords: MOG, NMOSD, MS, Demyelination, Autoantibody

1 Introduction

MOG is a highly conserved transmembrane protein of 28 kDa localized on the outermost surface of myelin sheaths in the central nervous system (CNS) (1). This localization makes the protein accessible for pathogenic autoantibodies. In many autoimmune disorders, disease-associated autoantibodies have emerged as important diagnostic biomarkers (2). It is known since more than 20 years that MOG is a target of demyelinating antibodies (abs) in experimental animals (3) and that only abs recognizing conformationally intact MOG can induce demyelination (4). Subsequent studies searching for abs to MOG in human patients were controversial: this can partially be explained by the use of different methods such as ELISA, Western blot, and cell-based assays (5).

A breakthrough was the discovery that a proportion of children with demyelinating diseases has high levels of autoantibodies against MOG detected by in vitro-translated and self-assembling radiolabeled tetramers (6). Numerous subsequent studies confirmed the presence of abs to MOG in children with demyelinating diseases and showed that also a cell-based assay with MOG-transfected cells is useful to identify these abs in pediatric patients (5, 7). Abs against conformationally intact MOG—as displayed on

the surface of transfected cells—occur in about 25 % of pediatric patients with MS and ADEM. While these abs tend to persist over time in pediatric MS, they appear only transiently in children with ADEM (8). Anti-MOG abs are found also in adults, namely in patients with anti-aquaporin-4 (AQP4)-negative neuromyelitis optica spectrum disorders (NMOSD) (9, 10), in optic neuritis (11), in very few patients with demyelinating syndromes associated with anti-NMDA-receptor abs (12) and in one patient with encephalomyelitis showing features overlapping with both MS and NMOSD (13).

Are human autoantibodies against MOG pathogenic? Although the pathogenicity of human abs to MOG has not yet been formally demonstrated in transfer experiments with affinity-purified or recombinant IgG and although the injection of concentrated human serum into rodents is difficult to interpret (14), there is good evidence that they are pathogenic: they are of the complement activating isotype IgG1 (8–10); IgG from children with anti-MOG abs induces cytoskeletal alterations in a human oligodendroglial cell line (15); intracerebral injection of IgG pooled from anti-MOG-positive NMO patients in mice resulted in transient pathology (16); the histopathology of an adult patient with abs to MOG and relapsing-remitting encephalomyelitis strongly resembles anti-MOG-mediated demyelination in animals and MS type II (13). Transfer attempts to experimental animals have to consider that only about ¼ of all human autoantibodies against MOG show cross-reactivity to rodent MOG (17).

In this chapter, we describe a flow cytometry assay that is used in our laboratory for the detection of serum anti-MOG abs in patients with demyelinating diseases of the CNS. The employment of this cell-based assay (Figs. 1 and 2) allows also to determine the epitopes recognized on conformationally intact MOG (13, 17).

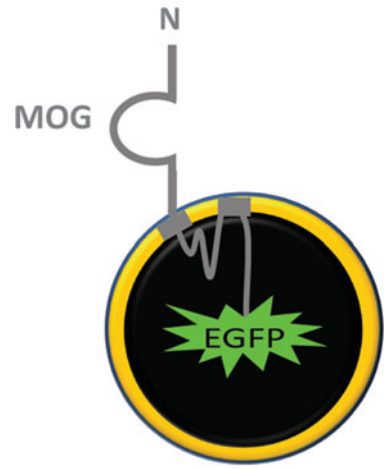

Fig. 1 Illustration of full-length MOG fused to EGFP as displayed by the transfected cells. The cartoon of MOG is based on Fig. 1b from (18)

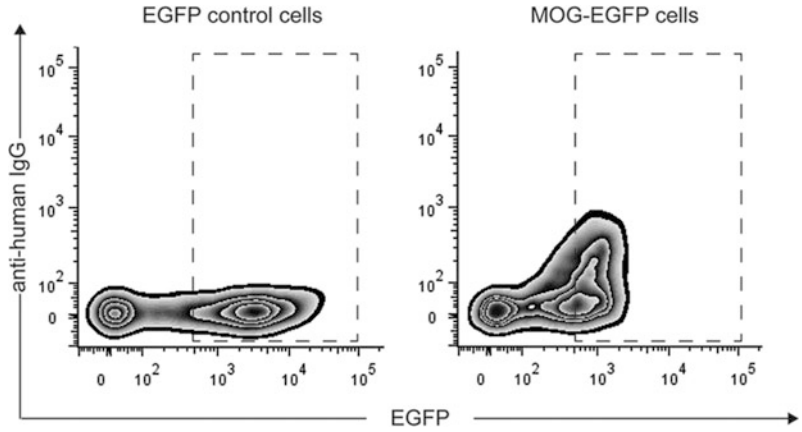

Fig. 2 Quantitative analysis of the anti-MOG response. FACS plots of HeLa cells transfected with EGFP alone (*left*) or with MOG-EGFP (*right*) that were incubated with a serum sample which scored positive for anti-MOG (FACS ratio 10.7). To determine the MOG reactivity, we gate on the cells inside the *dashed rectangle* and calculate the FACS ratio as MFI (MOG-EGFP)/MFI (EGFP only)

2 Materials

1. Cell culture facility: For the culture of HeLa cells we use Dulbecco's modified Eagle's medium supplied with 10 % fetal calf serum (FCS) and 1 % penicillin-streptomycin (Pen Strep).
2. Refrigerated centrifuge with swing rotor for 96-well plates.
3. Phosphate buffer saline (PBS), trypsin/EDTA, FACS buffer (1 % FCS in PBS).
4. Transfection reagent (Lipofectamine 2000®, Invitrogen, Carlsbad, California, USA).
5. Full-length wt MOG construct fused C-terminally to EGFP and control plasmid pEGFP-N1 (CLONTECH Laboratories, Mountain View, CA, USA) (*see* **Note 1**) (17).
6. Murine anti-MOG monoclonal antibody (mAb) (e.g., 8-18C5) as control of surface expression of MOG in the transfectants and F(ab')$_2$ Biotin-SP goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) (*see* **Note 2**).
7. Human serum samples to test for autoantibodies to MOG.
8. F(ab')$_2$ Biotin-SP-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA).
9. Propidium iodide for dead cell staining.
10. AlexaFluor® 647-conjugated Streptavidin (Jackson ImmunoResearch, West Grove, PA, USA).
11. Flow cytometer (e.g., BD Bioscience FacsVerse™, San Jose, CA, USA).

3 Methods

1. Keep cells growing in culture medium and collect them for the experiment when they are 80 % confluent.
2. Wash cells once with PBS and remove them from plate with trypsin/EDTA.
3. Suspend detached cells in culture medium and collect them in a 15 ml plastic tube.
4. Centrifuge for 5 min at 200 × *g* and suspend in 5 ml culture medium.
5. Seed cells at 1.5×10^5/ml in culture medium (*see* **Note 3**) and divide them into two cell culture dishes (for a 10 cm culture dish we use 10 ml of cell suspension).
6. Transfect cells in one dish with MOG-EGFP and cells in the other dish with EGFP when they are 90 % confluent.
7. Collect cells 24 h after transfection; remove them from plate as described in **steps 2–4**.
8. For determination of anti-MOG serum abs, transfer cells to a 96-well plate for flow cytometry. Use 50,000 cells/well.
9. Centrifuge for 5 min at 200 × *g* and wash cells by adding to the pellet 150 μl of FACS buffer. Repeat this step once more (*see* **Note 4**).
10. Incubate cells with a 1:50 human serum dilution in FACS buffer for 45 min at 4 °C. Cells used as a control for surface expression of the MOG construct are incubated with the mAb 8-18C5 (0.5 μg/ml).
11. Wash cells three times with FACS buffer as previously described and incubate them with a 1:500 dilution of the biotin-SP-conjugated goat anti-human or anti-mouse IgG for 30 min at 4 °C (*see* **Note 5**).
12. Wash cells as above and incubate them in the dark with a 1:2,000 dilution of AlexaFluor® 647-conjugated streptavidin for 30 min at 4 °C.
13. Wash three times, add to each cell pellet 100 μl of a 1:2,000 dilution of propidium iodide in PBS, and transfer them to FACS tubes.
14. Measure at least 20,000 events in a flow cytometer (*see* **Note 6**).
15. Perform analysis using a software such as FlowJo: exclude dead cells and determine values of the mean fluorescence intensity (MFI) in the APC channel (Fig. 2) (*see* **Notes 7** and **8**).

4 Notes

1. This assay allows to investigate also the role of MOG-glycosylation for recognition by autoantibodies. The only glycosylation site of MOG (at least in transfected HeLa cells) is N31 (17). The glycosylated part itself is typically not recognized by MOG-specific autoantibodies as seen with the non-glycosylated variant N31D (17). However, about 10 % of all patients with anti-MOG abs recognize the deglycosylated MOG better (13, 17).
2. Besides the mAb 8-18C5, also other antibodies such as the murine mAb Y11 (17) can be used to evaluate the surface expression of the MOG construct.
3. Cell concentration is optimized for HeLa cells. Adjustment may be required if another cell type is used.
4. Keep FACS buffer at 4 °C and perform from now on all steps on ice.
5. Include as negative controls cells labeled with the biotin-conjugated secondary antibody but without the human serum.
6. We use unfixed cells, since we cannot exclude at this stage that fixation of MOG-transfected cells interferes with the recognition in some patients.
7. For binding analysis, gate on cells with a 500-fold higher EGFP-fluorescence intensity than the nontransfected cells and determine for each serum sample the FACS ratio (MFI of MOG-EGFP transfected cells/MFI of EGFP-only transfected cells) (Fig. 2). Transient transfection and gating on the high-expressing cells yield a high sensitivity. The calculation of the MFI ratio (*see* also Fig. 2) is more robust than the MFI difference, since the patients have a different background.
8. Determine a cutoff value by performing the same assay with control samples.

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Myelinating Cultures: An In Vitro Tool to Identify Demyelinating and Axopathic Autoantibodies

Maren Lindner and Christopher Linington

Abstract

Myelinating cultures derived from embryonic spinal cord provide an invaluable tool to detect demyelinating and axopathic autoantibodies in clinical samples. A single preparation will provide a minimum of 200 individual cultures allowing 60 or more samples to be assayed in triplicate.

Keywords: Microdissection, Cell culture, Immunofluorescence microscopy, Demyelination, Axonal loss, Autoantibodies

1 Introduction

Autoantibody-dependent effector mechanisms contribute to the immunopathogenesis of multiple sclerosis (MS), but their specificity and clinical relevance remain controversial (1). Myelinating cultures derived from embryonic spinal cord (2) provide the basis of a rapid, robust, and sensitive bioassay to detect the presence of clinically relevant demyelinating and axopathic autoantibodies in patient derived immunoglobulin preparations (3). Crucially, this culture system offers several distinct advantages over other methodologies designed to identify pathologically relevant autoantibodies in MS. The most important is that this approach confronts the autoantibody repertoire with a complete array of myelin and axonal autoantigens in molecular domains identical to those found in vivo. This not only means these autoantigens are presented to antibodies in their native conformation, but also that intermolecular interactions are retained that might act limit or even enhance epitope accessibility within the CNS (4). The use of myelinated cultures to identify pathogenic autoantibodies in MS sera was first described over 50 years ago (5), but at that time its efficacy was limited due to lack of reproducibility and objective measures to quantify demyelination (6). Building on these pioneering studies we exploited subsequent advances in tissue culture techniques, imaging and data analysis to develop an in vitro assay to identify nanomolar concentrations of demyelinating or axopathic autoantibodies.

The remaining limitation of this approach is that antibody detection is dependent on recognition of a xenogenic target, a problem that will only be resolved once techniques are available to derive reproducibly myelinated cultures from human stem cells.

2 Materials

2.1 Stocks and Solutions

1. *Poly-L-Lysine (1.33 μg/ml)*
   Dissolve 25 mg of poly-L-lysine hydrobromide in 6.25 ml sterile water. Filter through a 0.22 μm filter and store in 66 μl aliquots at −20 °C.
2. *Hydrocortisone*
   Dissolve 100 mg of water soluble hydrocortisone in 27.6 ml sterile water (10 mM stock, store at −20 °C). Add 20 μl of 10 mM stock to 19.98 ml sterile water to create a 10 μM stock. Filter and store 500 μl aliquots at −20 °C. Use at an end concentration of 50 nM (250 μl of 10 μM stock in 49.75 ml DM).
3. *Biotin*
   Dissolve 100 mg Biotin in 100 ml sterile 1 M NaOH (1 mg/ml stock). For working stock (1,000×) dilute 1 ml in 99 ml sterile water. Filter and store 100 μl aliquots at −20 °C. Use at an end concentration of 10 ng/ml (50 μl of 10 μg/ml stock in 50 ml DM).
4. *Insulin*
   Dissolve 100 mg Insulin (bovine pancreas, Sigma) in 200 ml 10 mM HCl/H_2O (0.5 mg/ml stock). Filter and store at 2 ml aliquots at −20 °C. Use at an end concentration of 10 μg/ml (1 ml of 0.5 mg/ml stock in 49 ml DM).
5. *Collagenase*
   To make a 0.1 % solution, dissolve 20 mg collagenase type I in 20 ml Leibovitz L-15 media. Store in 500 μl aliquots at −20 °C.
6. *SD (Soybean Trypsin Inhibitor)*
   Add 13 mg trypsin inhibitor (soybean), 1 mg Dnase I (bovine pancreas) and 75 mg BSA fraction V to 25 ml Leibovitz L-15 media. Stir until fully dissolved, filter and store in 2 ml aliquots at −20 °C.
7. *30 % glucose*
   30 g glucose added slowly to 100 ml sterile water with constant stirring. Filter and store in 5 ml aliquots at −20 °C.
8. *7.5 % $NaHCO_3$*
   7.5 g $NaHCO_3$ added slowly to 100 ml sterile water. Filter and store in 5 ml aliquots at −20 °C.

9. *Putrescine*
   Make a 600 μM (9.6 μg/ml) stock solution by dissolving 96.6 mg putrescine in 100 ml sterile water and store at −20 °C.
10. *Selenium*
    Prepare a 3 mM stock solution by adding 1.93 ml sterile water to 1 mg selenium and store at −20 °C.
11. *Progesterone*
    Add 1 mg Progesterone to 1.59 ml 95 % Ethanol (629 μg/ml) and store at −20 °C.
12. *1 M HEPES*
    To 23.8 g HEPES add approximately 80 ml sterile water, dissolve, and then make up to a final volume of 100 ml. Filter and store in 3–5 ml aliquots at −20 °C.
13. *10× Hormone Mix*
    Add 5 ml *30 % Glucose*, 3.75 ml *7.5 % $NaHCO_3$*, 1.25 ml *1 M HEPES*, 250 mg *transferrin* (human, Sigma T1147), 6.25 ml *insulin* (human recombinant insulin, 9.5–10.5 mg/ml; Sigma I-9278) in 19.75 ml sterile water. Add 25 ml *putrescine* of 600 μM stock, final concentration 60 μM. Add 25 μl *selenium* of a 3 mM stock solution, final concentration 0.3 μM. Add 25 μl *progesterone* from stock solution, final concentration 0.0629 μg/ml. Fill up to 250 ml with *DMEM/F12 media* (Invitrogen, 21331-020), filter and aliquot in 25 ml, store at −80 °C.
14. *10× PBS*
    Dissolve 80 g NaCl (Sigma), 2 g KCl (Sigma), 2 g KH_2PO_4 (Sigma) and 11.8 g Na_2HPO_4 (Sigma) in approximately 800 ml sterile water and make up to 1 l.
15. *Blocking buffer* (*1 % BSA/10 % horse serum/PBS*)
    Dissolve 1 g BSA (Sigma) and 10 ml Horse serum (heat inactivated and filtered, Sigma) in 90 ml PBS. Store in 10 ml aliquots at −20 °C.
16. *4 % PFA*
    Dissolve 4 g Paraformaldehyde (PFA, Sigma) in 100 ml PBS on a heated plate under constant stirring. Filter and store in aliquots at −20 °C.
17. *0.5 % Triton X/PBS*
    Mix 250 μl Triton X (Sigma) with 50 ml PBS.
18. *Mowiol 4-88*
    Place 12 g Glycerol (Sigma) into a beaker, add 4.8 g Mowiol 4-88 (Calbiochem) and place on a stirring device. Add 12 ml distilled water and leave for 2 h at RT, then add 24 ml 0.2 M Tris pH 8.5 (0.2 M Tris is 2.42 g Tris base per 100 ml distilled water titrated to pH 8.5 with 10 N HCl) and warm to 50 °C stirring continuously until the Mowiol has dissolved

completely. Clarify by centrifugation at 400 × *g* for 20 min. Aliquot supernatant and store at −20 ° C; stable at least for 12 months. If necessary add DAPI (Sigma) to the Mowiol solution to a final concentration of 1 μg/ml.

19. *Other reagents*
Source of complement: rabbit sera (Sigma). Primary Antibodies for staining: myelin markers such as Z2 (anti-MOG, mouse IgG2a, own production), MBP (mouse IgG2a, 1:100, Millipore) or PLP (mouse IgG2a, 1:200, Millipore); axonal marker such as SMI31 (mouse IgG1, 1:1,000, Abcam). Fluorescence labelled secondary antibodies such as goat anti-mouse IgG1 Alexa Fluor 568 (Invitrogen) and goat anti-mouse IgG2a Alexa Fluor 488 (Invitrogen).

2.2 Cell Culture Media

1. *Neurosphere Media* (*NSM*)
Add 25 ml Hormone Mix (10×), 5 ml 30 % Glucose, 3.75 ml 7.5 % $NaHCO_3$, 1.25 ml 1 M HEPES, 2.5 ml L-Glutamine (Sigma), 2.5 ml Pen/Strep (Sigma) and 0.625 ml 4 % BSA in L15 media to 210 ml DMEM /F12.
2. *Astrocyte Media*
Add to 450 ml DMEM (low glucose, Invitrogen) 50 ml FBS (heat inactivated, filtered, Sigma), 2.5 ml L-glutamine, and 5 ml Pen/Strep.
3. *Plating Media* (*PM*)
Add to 25 ml DMEM (4.5 g/l glucose, 1 % Pen/Strep, 1 % Sodium Pyruvate) 12.5 ml Horse serum, 12.5 ml HBSS (Invitrogen), and 0.5 ml L-glutamine.
4. *Differentiation Media DM−*
Add to 49.5 ml DMEM (4.5 g/l glucose, 1 % Pen/Strep, 1 % Sodium Pyruvate) 50 μl Biotin (1 mg/ml), 250 μl N1 Supplement (100×, Sigma), and 250 μl Hydrocortisone (10 μM).
5. *Differentiation Media plus INSULIN DM+*
Add to 48.5 ml DMEM (4.5 g/l glucose, 1 % Pen/Strep, 1 % Sodium Pyruvate) 50 μl Biotin (1 mg/ml), 250 μl N1 Supplement (100×, Sigma), 250 μl Hydrocortisone (10 μM), and 1 ml Insulin.

2.3 Sample Preparation

To avoid effects mediated by other serum components the assay is best performed using purified immunoglobulin preparations. We routinely isolate IgG from clinical samples by Protein G affinity chromatography (GE Healthcare), whilst IgM can be using CaptureSelect™ IgM Affinity Matrix (Life Technologies). Purified immunoglobulins should be stored at 5 mg/ml in 1× PBS, pH 7.4 at −20 °C.

3 Methods

All procedures are done under sterile or semi-sterile (dissections) conditions. Dissections should be performed as quickly as possible and tissue and media should be kept on ice to avoid cell death. Once cells are in culture make sure you warm media to 37 °C before feeding. Since you are dealing with a long-term primary cultures always make sure you work under sterile conditions.

Coating Coverslips

1. Add 66 μl PLL to 20 ml sterile water in petri dish (90 mm).
2. Incubate coverslips (13 mm diameter) for 2 h at 37 °C.
3. Rinse three times with sterile water (*see* **Note 1**).
4. Dry coverslips in an upright position.

Generating Neurospheres

1. Dissect out the corpus striatum from P1 *Sprague Dawley* rat brains.
2. Place a maximum of four stria into a bijoux containing 1 ml ice cold Leibovitz L-15 media.
3. Triturate tissue using a glass Pasteur pipette and transfer to a 15 ml falcon tube. Note: 1 bijoux = 1 falcon tube = one flask.
4. Centrifuge for 5 min at ~140 × *g*.
5. Remove supernatant without disrupting the pellet.
6. Resuspend pellet in 2 ml NSM, add 4 μl EGF (Peprotech, 100 ng/ml stock) and transfer to a T75 flask containing 18 ml NSM.
7. Feed twice a week by addition of 5 ml NSM supplemented with 4 μl EGF.
8. It usually takes 5–7 days before the neurospheres are large enough to settle onto the bottom of the flask at which point they deemed ready to be harvested to generate astrocytes.
   Note: Striata from rats make three flasks of neurospheres, which is usually enough to make up eighteen 24-well plates of astrocytes.

Generation of Astrocyte Monolayers

1. Prepare 24-well plates by placing a PLL-coated coverslip in each well.
2. Transfer all flasks to 50 ml Falcon tubes, and centrifuge for 5 min at 500 × *g*.
3. Remove supernatant and resuspend cells in 12 ml astrocyte media per plate. Calculate how many 24-well plates you want to make and adjust volumes accordingly.

4. Constantly agitate the cell suspension gently, and add 0.5 ml to each well.
5. After all the cell suspension is pipetted out carefully add a further 0.5 ml astrocyte medium to each well.
6. Feed twice a week by removing half the media and replacing with fresh astrocyte medium.
7. It usually takes around 7 days for the astrocytes to form a confluent monolayer. Use an inverted phase-contrast microscope to make sure that the monolayer is confluent before use.

Spinal Cord Cells (*See* **Note 9**).

1. Use time-mated female rats to ensure obtaining E15.5 embryos.
2. Dissect out the gravid uterus, place in a dish containing ice cold HBSS.
3. Remove embryos from their embryonic sacs and transfer to a small Petri dish (35 mm).
4. Decapitate embryos taking care not to remove the cervical flexure.
5. Remove the top layer of skin above the spinal cord and open up the meninges.
6. Once the spinal cord is exposed, gently remove it from the embryo.
7. Remove meninges and dorsal root ganglia by holding the spinal cord and, starting at the cervical region, peeling back the meninges.
8. Place the spinal cord into a bijoux containing 1 ml ice cold HBSS.
9. Use a maximum of five spinal cords per bijoux.
10. Add 100 μl 2.5 % trypsin solution and 100 μl 1 % collagenase I per ml HBSS and incubate at 37 °C for 15 min.
11. Stop digestion by adding 2 ml of SD, gently shaking and letting the dissociated tissue sink to the bottom of the bijoux.
12. Aspirate some media and triturate the remaining 1–2 ml into a 15 ml tube.
13. Centrifuge 800 × *g* for 5 min at 4 °C.
14. Remove supernatant and resuspend all pellets in 1 ml of PM.
15. Make up to 10 ml in PM.
16. Count cells and dilute to 3×10^6 cells/ml (use 50 μl per coverslip = 150,000 cells/coverslip).
17. Place up to three PLL coated coverslips in a 35 mm diameter Petri dish.

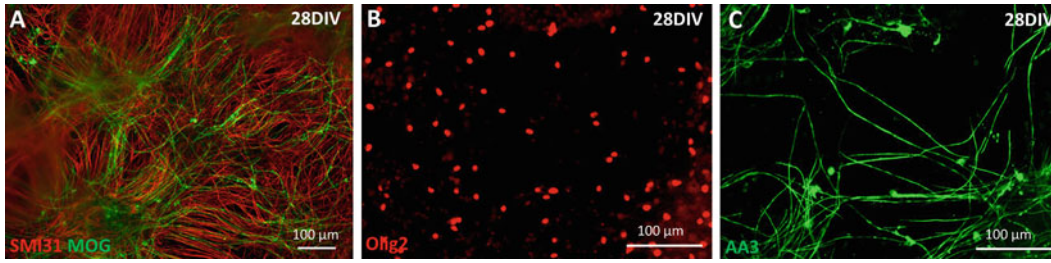

Fig. 1 Representative immunofluorescence images of myelinating cultures (28 DIV). (**a**) Two colour immunofluorescence microscopy reveals the presence of a dense bed of neurites (*Red*; neurofilament-specific antibody SMI31) and myelinating oligodendrocytes (*Green*; myelin oligodendrocyte glycoprotein-specific antibody Z2). Note only a relatively small proportion of neurites are myelinated and myelin sheaths are not present along their full length. (**b**) Cells of the oligodendrocyte lineage can also be identified using antibodies specific for the transcription factor Olig2 (*Red*). (**c**) Representative immunofluorescence image obtained using the PLP/DM20-specific antibody AA3 (*Green*)

18. Carefully pipette 50 µl of the spinal cord cell suspension onto each coverslip.
19. Incubate at 37 °C for 2 h to allow them to start attaching to the substrate.
20. Mix 60 ml DM^+ with 45 ml PM media.
21. Carefully add 1.050 ml of this medium to the petri dishes.
22. Culture at 37 °C, CO_2 7.5 %.
23. Feed three times per week by removing 500 µl supernatant and replacing with 600 µl media.
24. Feed with DM^+ until 12 DIV thereafter use DM^-.
    Note: One set of embryos should provide approximately 240 coverslips.

Antibody-Mediated Demyelination/Axonal Loss

1. To test for demyelinating/axopathic antibodies use myelinated cultures 25–28 DIV (*see* Fig. 1).
2. Confirm cultures are well myelinated: axonal density should be at least 70 %, myelination >8 % (*see* **Notes 3** and **9**).
3. Add test sample in the presence and absence of rabbit serum as a source of complement (*see* **Notes 2** and **4**–**8**).
4. Incubate for 16 h at 37 °C in 7.5 % CO_2.
5. For fixation, staining, and analysis protocols see below.

Monitoring Changes Induced by Soluble Factors Over Time

1. To assess effects on myelination cultures are treated with the factor of interest diluted in culture medium from 18 to 28 DIV (*see* **Note 3**).

2. To investigate effects on neurite outgrowth or OPC numbers the experiment should be initiated at time points between 6 and 12 DIV.
3. Feed cultures three times a week with appropriate medium in the presence or absence of the factor of interest.
4. Harvest coverslips at different time points. For fixation, staining and analysis protocols see below.
5. To assess effects on cells of the oligodendrocyte lineage use appropriate combinations of stage-specific markers to identify early and late progenitors (Olig2, A2B5, PDGFRα, NG2, O4), pre-myelinating oligodendrocytes (PLP/DM20), and terminally differentiated/myelinating oligodendrocytes (MOG) (*see* **Note 9**).

Fixation and Staining

1. Fix cells by putting coverslips directly into 4 % PFA for 20 min after which they are washed three times in PBS.
2. Permeablize cells with 0.5 % Triton X100 for 10 min.
3. Wash three times in PBS.
4. Incubate in blocking buffer for 60 min.
5. Incubate with primary antibodies diluted in blocking buffer for 45 min.
6. Wash three times in PBS.
7. Incubate with appropriate fluorophore-conjugated species/isotype-specific secondary antibodies diluted in blocking buffer for 15 min in the dark.
8. Wash three times in PBS.
9. Rinse in distilled H_2O.
10. Mount coverslips in Mowiol + DAPI.
11. Leave to harden overnight in the dark.
12. Further store in the dark at 4 °C.

Quantification

1. To analyze axonal density and myelination take ten pictures per coverslip (three coverslips per condition) at a 10× magnification with a fluorescence microscope. To analyse changes in cell number we recommend 20× magnification).
2. Axonal density is calculated using ImageJ software (NIH systems, version 1.41o) by expressing the SMI-31$^+$ area as percentage of the total field area.
3. To calculate the percentage of myelinated axons, immunoreactivity associated with MOG$^+$ or PLP$^+$ myelin sheaths is measured using ImageJ after they are manually highlighted using Adobe Photoshop Elements 4.0 (brush shape size 9).

4. Percentage myelin is then calculated by dividing the area of highlighted myelin by axonal density.
5. To quantify cell numbers, a minimum of 30 images is taken from three cover-slips (20× magnification) and immunopositive cells are counted using the ImageJ cell counter function (3).
6. Alternatively, pictures can be quantified using CellProfiler cell image analysis software (7). Pipelines for myelinating cultures designed by our group can be found at https://github.com/muecs/cp.

4 Notes

1. After coating wash coverslips extensively as PLL is toxic.
2. If you screening sera rather than purified immunoglobulins for demyelinating or axopathic activity they must be heat inactivated before use to abolish endogenous complement activity.
3. Screen untreated cultures before starting and when terminating experiments to be absolutely sure that they satisfy your criteria such as neurite density, myelination, and OPC numbers as appropriate for your research question.
4. Always test the activity of your complement source. Complement is very sensitive to freeze thawing, and activity is lost if stored at 4 °C. In addition high concentrations of rabbit sera can induce demyelination and/or axonal loss.
5. Optimal dilutions for each antibody must be determined before they are used experimentally.
6. It is essential to include appropriate negative and positive controls each time cultures are used to assay for demyelinating or axopathic antibodies. This should include:
   (a) Immunoglobulin preparation alone
   (b) Complement source alone
   (c) Demyelinating monoclonal antibody alone, e.g., mAb O4 10 μg/ml
   (d) Demyelinating monoclonal antibody plus complement source
   (e) Untreated cultures
7. Immunoglobulin preparations should be tested at several concentrations up to 500 μg/ml in the presence or absence of a source of complement.
8. Sera are screened routinely at a dilutions between 1:30 and 1:400.

9. Extent of myelination varies between preparations, but this can be minimalized by ensuring:
   (a) Clean accurate and rapid tissue dissections
   (b) Seeding spinal cord cells onto *fully confluent* astrocyte monolayers
   (c) Take care not to disturb the cultures when feeding
   (d) Always use freshly made up media.
   (e) The activity of the 10× Hormone Mix declines even when stored at −80 °C. Keep no longer than 6 months.

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Part IV

Imaging Techniques

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In Vivo Visualization of (Auto)Immune Processes in the Central Nervous System of Rodents

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Abstract

The CNS is effectively shielded from the periphery by the blood–brain barrier (BBB) which limits the entry of cells and solutes. However, in autoimmune disorders such as multiple sclerosis, immune cells can overcome this barrier and induce the formation of CNS inflammatory lesions. Recently, two-photon laser scanning microscopy (TPLSM) has made it possible to visualize autoimmune processes in the living CNS in real time. However, along with a high microscopy standard, this technique requires an advanced surgical procedure to access the region of interest. Here, we describe in detail the necessary methodological steps to visualize (auto)immune processes in living rodent tissue. We focus on the procedures to image the leptomeningeal vessels of the thoracic spinal cord during transfer experimental autoimmune encephalomyelitis in LEW rats (AT EAE) and in active EAE in C57BL/6 mice (aEAE).

Keywords: Dura (mater) spinalis, Experimental autoimmune encephalomyelitis (EAE), Encephalitogenic T cells, Laminectomy, TPLSM (two-photon laser scanning microscopy), Spinal cord window, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG)

1 Introduction

Cells of the immune system in particular leukocytes are in constant movement in order to perform their immune defense function. However, this essential and unique aspect was barely experimentally addressable until, 12 years ago, two-photon laser scanning microscopy (TPLSM) was introduced into the immunological field (1, 2). The technical details of TPLSM were excellently reviewed elsewhere (3, 4). Essentially this technique allows long-term high-resolution recording of immune cell dynamics in situ, in living milieu with minimal photo-bleaching and phototoxicity. Therefore, it rapidly became the standard technique for in vivo imaging of immune processes (5). When applied to the neuroimmunological field, this new technological approach made it possible to obtain first initial insights into the dynamics of autoimmune processes within CNS tissue (6–10). For example, in a rat model of multiple sclerosis, it was possible to show that once the encephalitogenic T cells arrive at the BBB, they crawled extensively along the inner surface of leptomeningeal vessels, mainly against the blood

flow (11). After diapedesis the T cells kept crawling along the extraluminal side, hereby encountering local phagocytes able to present autoantigen to the encephalitogenic T cells. As a consequence, these myelin-reactive effector T cells were strongly reactivated and produced pro-inflammatory cytokines (12). These interactions between effector T cells and APCs were shown to be crucial for the further invasion of the parenchyma (11, 12). However, an exhaustive understanding of how T cells enter the brain and what molecular cues guide them to the target organ is far from being achieved. Furthermore, the contribution and behavior of other immune cells besides T cells within the autoimmune process are still unclear, demanding further TPLSM studies.

In this chapter we will describe the procedures to visualize the initiation of the autoimmune processes in the leptomeningeal vessels of the spinal cord in two different rodent models of multiple sclerosis: experimental autoimmune encephalomyelitis in LEW rats induced by passive transfer of myelin-specific T cells (AT-EAE) and EAE in C57BL/6 mice induced by active immunization with MOG peptide (aEAE). In both of these "classical" EAE models, the animals develop extensive autoinflammatory lesions of the CNS that are preferentially located in the lumbar/sacral spinal cord. The clinical picture is characterized by ascending pareses. It is well established that autoreactive, antigen-specific $CD4^+$ T cells are responsible for the disease pathogenesis in these EAE models. For a detailed description of EAE models, we refer the interested reader to recent excellent reviews (13–15). Here, a particular emphasis will be placed on the surgical technique for exposing the spinal cord correctly and on the methods for monitoring the vital parameters of the animals.

2 Materials

In vivo two-photon imaging of autoimmune T cells in the rat spinal cord during adoptive transfer EAE.

2.1 Animals

8–10-week-old LEW rats.

2.2 Cells

Myelin-antigen-reactive effector T cell blasts genetically manipulated to stably express fluorescent protein.

2.3 Anesthesia

1. Xylazine.
2. Ketamine.
3. Isoflurane.

2.4 Reagents

1. 0.9 % sodium chloride solution.
2. Low-melting agarose.
3. Medical oxygen.
4. Fluorescence-conjugated dextran.

2.5 Surgical Devices

1. Stereomicroscope with fiber-optic illumination.
2. Tungsten carbide bur, round.
3. Tungsten carbide bur, round.
4. Dental micromotor.
5. Catheter tubes.
6. Pet trimmer.
7. Fine iris scissors, straight.
8. Vannas spring scissors, straight.
9. 2× Narrow pattern forceps, serrated tips.
10. Slim tissue forceps, 1 × 2 teeth.
11. Dumont medical forceps, curved shanks, serrated tips.
12. Dumont forceps, straight shanks, superfine tips.

2.6 Devices

1. Isoflurane vaporizer.
2. Single animal volume-controlled ventilator.
3. Patient monitors to control respiratory gases/anesthesia.
4. Pulse oxymeter.
5. Custom-built microscopy stage with a stereotaxic frame optimized for the fixation of rat vertebras.
6. Heating plate.
7. Thermoprobe.
8. Custom-built thermocontroller.
9. Infusion pump.

2.7 TPLSM Imaging Setup

1. LSM710/Axio Examiner.Z1 confocal microscope.
2. >2.5 W Ti:Sapphire Chameleon Vision II Laser device.
3. 20× water NA1.0 immersion objective W Plan Apochromat.
4. Software-controlled motorized stage.
5. Non-descanned detectors.
6. Filter cubes.
7. Band-pass filters: 442/46 nm, 525/50 nm, and 624/40 nm band-pass filters.
8. Acquisition software.

2.8 Image Processing and Raw Data Analysis

1. Computer workstation with high graphics processing capability.
2. Software solution for processing 4D raw data sets.

In vivo two-photon imaging of autoimmune T cells in the mouse spinal cord during active EAE.

2.9 Isolation and Adoptive Transfer of Naive MOG-Specific T Cells

1. GFP^+ 2D2 donor mice (16, 17) and WT recipients, both of C57BL/6 genetic background.
2. 40 μm mesh.
3. Mouse T cell enrichment kit.
4. PBS, EH medium (25 mM HEPES, DMEM, pH = 7.4).

2.10 Active EAE Induction in Mice

1. 1 mg/ml myelin oligodendrocyte glycoprotein-35-55 peptide (MOG_{35-55}, MEVGWYRSPFSRVVHLYRNGK).
2. Incomplete Freund's adjuvant.
3. Freeze-dried inactivated bacilli of Mycobacterium tuberculosis strain *H37Ra*.
4. Pertussis toxin.
5. 1 or 2 ml glass tuberculin syringes with Luer-Lock and a custom-built connecting piece built from two cannulas made of stainless steel.

2.11 Anesthesia

1. Xylazine.
2. Ketamine.
2. Dorbene vet (Medetomidin).

2.12 Surgical Devices

The same instruments as described for rat spinal cord preparation are used (*see* Section 2.5). The main difference is that the custom-built stereotactic frame is optimized for the lateral fixation of the mice vertebras.

2.13 TPLSM Imaging Setup, Image Processing

See Sections 2.7 and 2.8.

3 Methods

In vivo two-photon imaging of autoimmune T cells in the rat spinal cord during adoptive transfer EAE.

3.1 Generation of Myelin-Reactive Effector T Cells and Induction of Transfer Experimental Autoimmune Encephalomyelitis (AT-EAE)

1. The generation of myelin–basic-protein-reactive GFP^+ T cells ($T_{MBP\text{-}GFP}$ cells) has been previously described in detail (18). In brief, use 200 μg MBP in complete Freund's adjuvant containing 2 mg/ml inactivated *M. tuberculosis* for subcutaneous immunization of 6–8-week-old female LEW rats. Subcutaneous immunization is performed at the popliteal cavity and at the tail base.
2. Isolate T cells from inguinal, para-aortic, and popliteal lymph nodes 10 days post immunization.
3. Co-culture 2×10^6 lymphocytes per ml together with 1.5×10^5 GP + E86 packaging cells per ml in 96-well plates

in the presence of 10 μg/ml MBP. Packaging cells are stably transduced by a murine stem cell retrovirus (pMSCV) containing the eGFP cassette.

4. Two days afterwards expand lymphocytes in IL-2-containing growth medium.
5. Seven days after the primary culture, stimulate T lymphocytes with 10 μg/ml MBP in the presence of 14×10^6 irradiated (30 Gy) thymocytes per ml. Thymocytes serve as antigen-presenting cells (APCs).
6. Select T cells with appropriate antibiotics and further amplify them as reported in detail (18).
7. Following 2 days of restimulation, inject 5×10^6 activated $T_{MBP\text{-}GFP}$ cell blasts in a volume of 1 ml per animal into the tail vein of ether-anesthetized 6–8-week-old LEW rats. The cell suspension is typically in EH buffer (pH = 7.4) and kept on ice.
8. According to the scientific question and your experimental setup, spinal cord preparation can be performed at various time points following adoptive cell transfer. Note: for visualization/analysis of effector T cell behavior within leptomeningeal blood vessels, we perform TPLSM at approximately day 2–2.5 post transfer.

3.2 Preparation of Animals for CNS Imaging

1. Induce anesthesia in rats by intramuscular injection of 10 mg/kg xylazine combined with 50 mg/kg ketamine. Assess the anesthetic depth before proceeding: withdrawal reflexes (toe, paw, and ear pinch) and palpebral reflexes must be absent.
2. Tracheally intubate the animal. For this purpose, place the animal in supine position on the preheated microscopy stage. Secure the animal by using tapes fastened to the upper extremities. Perform a small vertical midline incision of the skin of the neck (below the cricoid cartilage). Carefully expose the upper trachea (from the cricoid cartilage down to the fourth ring or lower) by dissecting the surrounding dorsal and lateral connective tissue and muscles. Avoid bleeding as far as possible. Carefully perform a horizontal midline incision of the trachea between the second and third ring below the cricoid cartilage. Intubate the animal by carefully inserting the tip of a catheter of suitable size into the trachea. Slowly advance the tube approximately 5 mm further down the trachea. Remove tapes from the upper extremities and place the animal in prone position on the microscopy stage. Be careful not to dislocate the tube while connecting the animal to the ventilation system.
3. In order to administer fluids during the imaging session, cannulate the tail vein by inserting a catheter.

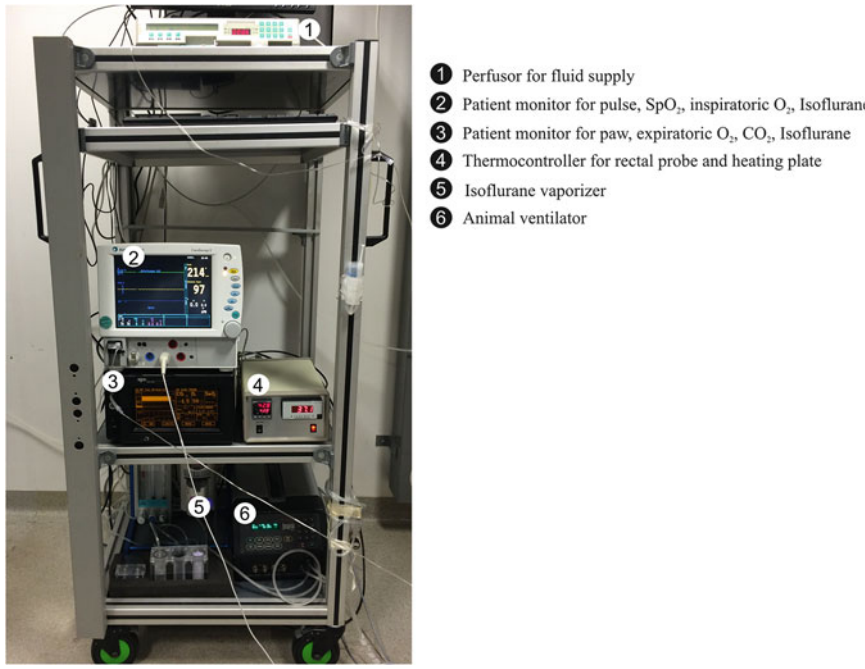

Fig. 1 Ventilation system/monitoring setup. A typical setup for intravital microscopy is indicated. Animals are supplied with medical oxygen and isoflurane via a volume-controlled animal ventilator. Physiological parameters are continuously checked via patient monitors and a thermocontroller. Fluid supply is ensured during the entire experiment by an infusion pump

3.3 Ventilation System and Monitoring Setup

During the entire imaging session, the animal is connected to a rebreathing anesthesia circuit supplied by medical oxygen and pressurized air, routed through an isoflurane vaporizer. Through this system the deep plane of anesthesia and the vital parameters are constantly controlled (Fig. 1). The animal is ventilated by a single animal volume-controlled ventilator with a tidal volume modifiable from 0.1 to 100 ml. The ventilation rate is adjusted according to the weight of the animal. In this way, spontaneous breathing can be reduced to a minimum and breathing artifacts are negligible. During the imaging, inspired and expired O_2, CO_2, and isoflurane can be assessed by using patient monitoring devices. Connect a feedback-regulated rectal thermoprobe to maintain the body temperature between 37 and 37.5 °C. Additionally use a pulse oxymeter to monitor the animal's pulse and blood oxygen saturation (>95 %). Fluid supply (approximately 0.5 and 0.8 ml/h) during surgery and the imaging experiment can be ensured by using an infusion pump.

3.4 Surgical Procedure: Spinal Cord Window Preparation

1. Use a pet trimmer to shave off a patch of the animal's fur at the level of the thoracic spinal cord. Be sure that no hair fragments remain at the desired spot since these will create strong

autofluorescence. For sterile surgery use 70 % ethanol to disinfect the skin before proceeding and use autoclaved instruments.

2. Perform a midline skin incision of 2–3 cm at the level of the thoracic spinal cord using straight fine iris scissors and straight forceps with serrated tips.

   All the following steps should be performed by using a stereomicroscope with fiber-optic illumination for optimal specimen visualization during surgery.

3. Dissect the paravertebral musculature (*m. erector spinae*) from the exposed vertebras using straight serrated tip forceps and straight spring scissors. Control bleeding with gauze. Be careful not to touch the intervertebral space.
4. Fixate three contiguous vertebras laterally (at the level of the pedicle) with steel needles in a rigid custom-built frame. This frame can be directly mounted on the microscopy stage (Fig. 2a, b). For this procedure it may be helpful to use slim tissue forceps to carefully grab the spinous processes of the exposed vertebras. Ensure that the vertebras are properly fixated before proceeding.
5. Use a vibration-free dental micromotor with a round tungsten carbide bur to flatten the spinous process of the central vertebra. Be sure to frequently flush the region with 0.9 % isotonic sodium chloride solution to avoid heating during drilling.
6. Create a ring of low-melting agarose (2.5 % in 0.9 % isotonic sodium chloride solution) around the flattened central vertebra. Be careful to ensure that the temperature of the agarose does not exceed 37–38 °C.
7. Perform a laminectomy by using a dental micromotor with a fine round tungsten carbide bur to incise two sagittal grooves in a distance of at least 3 mm away from the lateral edges of the vertebra. Immediately afterwards, flush the region with 0.9 % isotonic sodium chloride solution to prevent dehydration of the tissue.
8. Use medical forceps with curved, serrated tips to grip the far edges of the loosened bone flap. Be careful while peeling the latter away from the underlying dura (mater) spinalis. Carefully flush the exposed spinal cord with 0.9 % isotonic sodium chloride solution. In this way, the integrity of the agarose ring can be easily checked.
9. Carefully remove the dura (mater) spinalis by using straight forceps with ultrafine tips. Be careful not to perforate the underlying arachnoidea (mater) spinalis, especially during tissue swelling due to (auto)immune inflammation (Fig. 2b). Control bleeding carefully with gauze without generating any pressure on the underlying tissue.

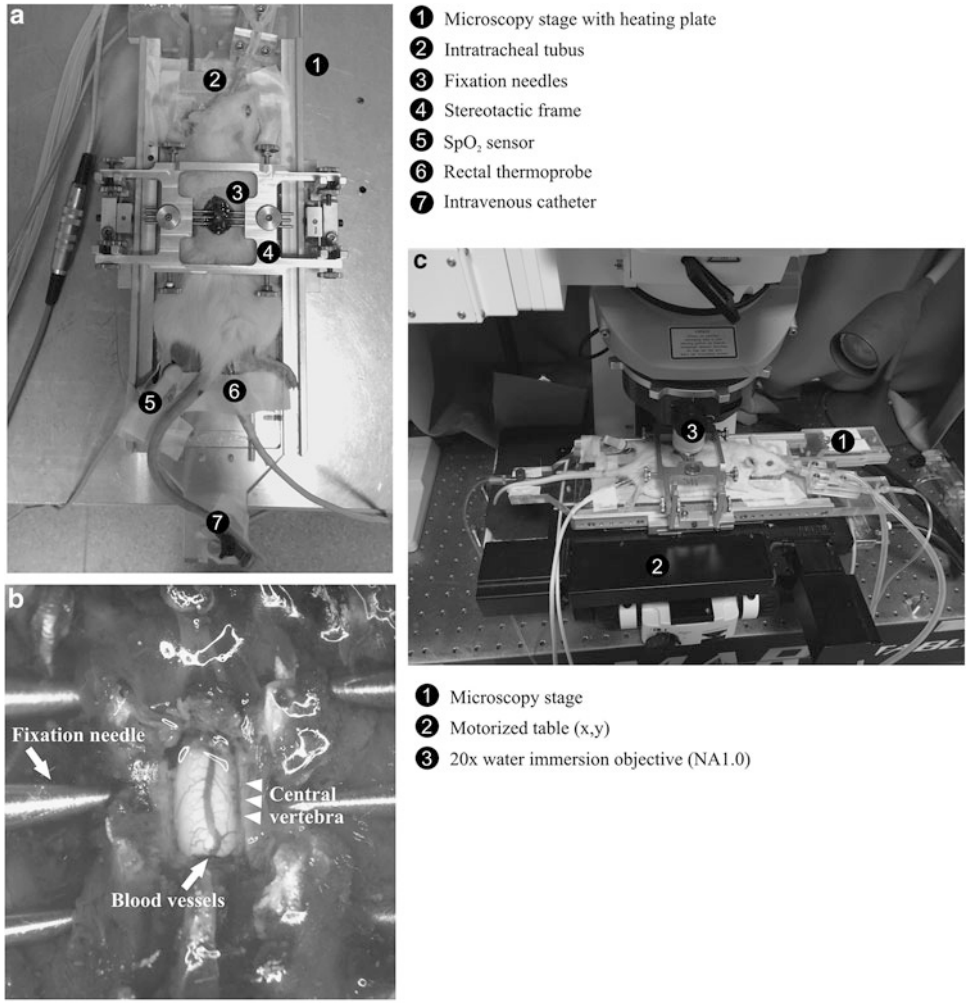

Fig. 2 Preparation of a spinal cord window for intravital TPLSM. (**a**) Indicated is a LEW rat in prone position on a heated custom-built microscopy stage. The animal is intratracheally intubated and ventilated. Physiological conditions are assessed by a rectal thermoprobe connected to a thermocontroller (not shown) and an SpO_2 sensor attached to the left leg. Three thoracic vertebras are fixated laterally by steel needles within a custom-built stereotactic frame. Fluid supply is ensured via an intravenous catheter at the tail. (**b**) Detailed view on a fixated, laminectomized thoracic vertebra with exposed spinal cord channel in the center. Since the dura (mater) spinalis is removed, the subarachnoideal blood vessels are visible. (**c**) Side view of an animal on the microscopy stage mounted on a motorized (*x*, *y*) table. A 20× water immersion objective is located above the spinal cord window

10. Transfer the microscopy stage carefully onto a motorized (*x*, *y*) table and place a water immersion objective above the spinal cord window (Fig. 2c).
11. Select a spot of interest under the microscope and start TPLSM. Clean the spinal cord window frequently by exchanging the isotonic sodium chloride solution. Ensure that animal parameters are reflecting physiological conditions during the entire experiment.

3.5 Two-Photon Laser Scanning Microscopy (TPLSM) for Monitoring the Behavior of Encephalitogenic GFP$^+$ Effector T Cells Within the Lumen of Leptomeningeal Blood Vessels

Here we use an upright LSM710/Axio Examiner.Z1 microscope combined with a >2.5 W Ti:Sapphire Chameleon Vision II Laser device. The excitation wavelength of the laser is software-controlled and tunable from 690 to 1,040 nm. This allows a flexible handling according to the experimental setup. In the following a typical experimental design is described as an example.

1. For visualization of leptomeningeal blood vessels, inject a 400 μg/kg fluorescence-conjugated dextran (e.g., Texas Red dextran, 70,000 MW) intravenously directly before starting TPLSM.
2. Start the acquisition software and tune the laser wavelength to 880 nm. Here, filter cubes are equipped with 442/46 nm, 525/50 nm, and 624/40 nm for simultaneous detection of collagen (by second harmonic generation), eGFP, and Texas Red dextran, respectively.
3. Set the stack height by scanning through the tissue in *z*-direction. For time-lapse recordings, z-stacks should be kept as minimal as possible as the number of z-sections correlates directly with the scanning time and therefore with the temporal resolution of the image. Here, we typically use stacks between 100 and 150 μm, with an approximate single stack size of 4–6 μm. For high-quality imaging, the resolution of the single *x*, *y* planes should be at least 512 × 512 pixels (here, 424.27 × 427.27 μm). Thereby, the acquisition rate during bidirectional scanning is approximately 1.3 s per *z*-plane including two times line averaging. Importantly, for reproducible motility analyses, the interval time has to be kept at an exact value. We typically record 30 min time-lapse videos composed of 58 cycles and 32 s time intervals between two cycles.
4. Start recording several spots. Ensure that the selected spots are comparable according to cell numbers, vessel size, etc.
5. Obtain 2D movies by generating maximum intensity projections with a suitable software solution and store them typically as .avi or .tiff series for further processing, e.g., modulate brightness and contrast, stabilization, cell counting, etc.
6. Analyze 4D (3D + time) raw data with a suitable software solution. Automated tracking modules can be used to trace individual cells within a 30 min recording interval. Note: according to your raw data, it might be essential to manually revise obtained trajectories.
7. Calculate motility parameters (velocity, track duration, track length, direction of movement, etc.) from the coordinates obtained by the software (Fig. 3). We define vascular crawling cells as individual lymphocytes that move in an amoeboid manner strictly within the lumen of a leptomeningeal blood

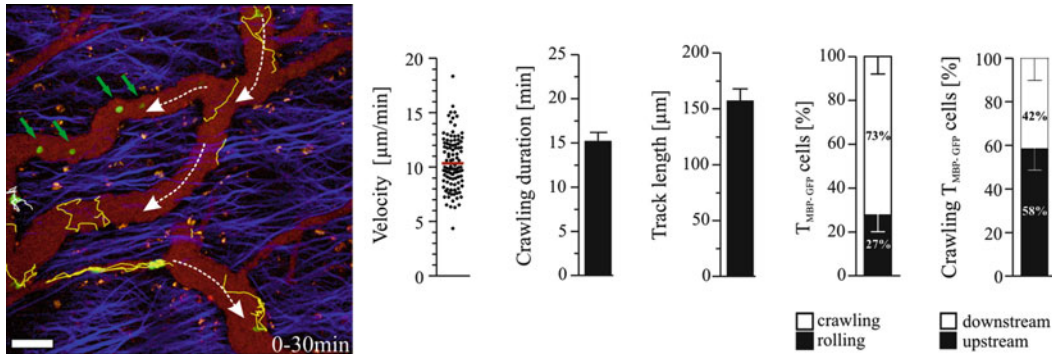

Fig. 3 Intraluminal locomotive behavior of $T_{MBP\text{-}GFP}$ cells within the lumen of leptomeningeal blood vessels. *Picture*: Shown is a representative intravital TPLSM recording of spinal cord leptomeningeal blood vessels 2.5 days p.t. Trajectories (*yellow*, intraluminal; *white*, extraluminal) of $T_{MBP\text{-}GFP}$ cells within a 30 min recording period are depicted. Rolling $T_{MBP\text{-}GFP}$ cells appear as *dots* (*green arrows*). *Blue*: Collagen signal via second harmonic generation. Scale bar: 50 µm. *Graphs*: Analyses are based on 30 min intravital TPLSM imaging data acquired during the early phase of EAE (d2.5 p.t.). Bars represent, from *left* to *right*, average velocity, average track duration, average track length of intraluminal crawling $T_{MBP\text{-}GFP}$ cells, proportion of intraluminal crawling vs. rolling $T_{MBP\text{-}GFP}$ cells, and proportion of $T_{MBP\text{-}GFP}$ cells crawling in (downstream) or against (upstream) the direction of the blood flow. Representative data from at least three independent experiments are depicted

vessel (*therefore, 4D analysis is essential!*) and visible for more than two framaes. In contrast, rolling lymphocytes are defined as several round-shaped dots moving strictly with the direction of the blood flow as described (11). Correlation between both migratory phenotypes is performed by quantifying absolute numbers of crawling versus rolling cells within an observation period of 30 min (Fig. 3).

In vivo two-photon imaging of autoimmune T cells in the mouse spinal cord during active EAE.

3.6 Isolation and Adoptive Transfer of Naive MOG-Specific T Cells

Isolate spleens and lymph nodes from GFP^+ 2D2 mice and prepare a single cell suspension by forcing organs through a 40 µm mesh into the EH medium using a plunger from a disposable 5 ml syringe. $CD3^+$ T cells can be obtained by a negative immunomagnetic selection using a mouse T cell enrichment kit, according to the manufacturer's instructions. Confirm cell purity by flow cytometry. Typically more than 90 % of cells should be $CD3^+$. Resuspend naive T lymphocytes at a concentration of ca. $2–7 \times 10^6$ cells in 200 µl PBS and administer via the tail vein into the adult C57BL/6 wild-type mice (minimum 10 weeks of age).

3.7 Preparation of Immunization Emulsion

1. Prepare 1 mg/ml of complete Freund's adjuvant (CFA) by mixing inactivated *Mycobacterium tuberculosis* H37Ra with IFA. We recommend preparing a 4 mg/ml stock solution, which can be stored at 4 °C and diluted with IFA to 1 mg/ml working solution.

2. Prepare 1 mg/ml MOG_{35-55} in PBS.
3. Per mouse you will need to mix 50 µl of 1 mg/ml MOG_{35-55} peptide with 55 µl (1 mg/ml) CFA. We find that by taking 10 % more volume CFA than the antigen ensures the preparation of a completely homogenous mixture. Leave the emulsion for ca. 1 h at 4 °C and ensure that the emulsion is completely homogenous before immunization.

3.8 Active Induction of EAE

To induce EAE, immunize mice 2 days after adoptive transfer of T cells. Anesthetize mice by an i.p. injection of 80 mg/kg ketamine and 8 mg/kg xylazine and immunize s.c. into the tail base (50 µl each side). Inject 200 µl PTX (200 ng per mouse per day) on the same day of immunization and then 2 days after immunization. Mice usually get sick on d10–d14 post immunization.

3.9 Surgical Procedure for Intravital Imaging in the Spinal Cord

For intravital imaging, anesthetize immunized mice by an i.p. injection of a mixture of dorbene and ketamine to achieve a dose of 1 mg/kg body weight for dorbene and 100 mg/kg for ketamine in PBS. If you use injectable anesthetics for mice instead of isoflurane as described for rats above, you will not need to intubate the animal. Instead you can place an oxygen mask in front of the animal's snout. Ensure deep plane of anesthesia before proceeding with surgery by checking paw pinch reflex. The initial anesthetic dose is in general sufficient for a surgical plane of anesthesia for at least 1 h. Monitor the level of anesthesia for the whole experiment (surgery and imaging) and supplement with half of the initial dose i.p. if you observe a pinch withdrawal reflex (usually every 60 min). Perform microsurgical preparation for intravital microscopy as described above for rats (*see* Section 3.4).

3.10 Two-Photon Laser Scanning Microscopy (TPLSM)

The imaging acquisition is performed as described in Section 3.5.

4 Notes

In vivo two-photon imaging of autoimmune T cells in the rat spinal cord during adoptive transfer EAE.

1. *Mean airway pressure (paw) is elevated while the expiratory CO_2 is decreasing.*

   Probably the tube is blocked by saliva, clotted blood, etc. In this case, disconnect the animal from the ventilation and try to clean the intratracheal tube as fast as possible. To do this it might be helpful to use a superfine polyethylene tube connected to a perfusion pump that can be inserted within the intratracheal tube.

2. *Both airway pressure and CO_2 level are elevated.*

   Check whether the animal is breathing spontaneously. In this case check whether your anesthesia status is still sufficient. If not, increase the isoflurane supply carefully and/or reinject anesthesia intramuscularly.

3. *Decreasing stability of the recording during acquisition.*

   Carefully check whether the fixation needles are still decently adjusted to the vertebra. If not, try to rectify fixation. In some cases it might be necessary to redo the agarose ring. If so, ensure that the agarose temperature does not exceed 37–38° and avoid any contact with the exposed spinal cord channel. During the entire procedure keep the tissue moistened in order to prevent dehydration.

4. *Signal loss during TPLSM and reduction of imaging quality.*

   In this case stop acquisition and carefully clean the spinal cord window with gauze. Exchange the buffer with 0.9 % isotonic sodium chloride solution. If the problem persists, check (if possible) whether the laser beam path is correctly adjusted or whether the power of the external laser source is still sufficient.

In vivo two-photon imaging of autoimmune T cells in the mouse spinal cord during active EAE.

5. *EAE susceptibility.*

   Several factors can influence the EAE susceptibility in mice. Stress is one important factor, among others. If mice are transported from one facility to another prior EAE induction, you should allow at least few days after the transport for mice to adapt to the new facility before immunization. Furthermore, it is important to keep mice under optimal and constant temperature and humidity conditions. EAE susceptibility in mice can vary greatly from strain to strain and from lab to lab. Therefore, you might need to increase the dosage of the antigen for efficient induction of active EAE.

6. *Choosing the right dyes.*

   For a successful in vivo tracking of MOG-specific T cells, it is important that T cells express a fluorescent protein which is bright and photostable. Synthetic dyes (such as CFSE and CMTMR) often used for lymphocyte labeling for intravital imaging in the lymph node cannot be used in this setting because of an extensive proliferation of T cells during the priming phase and dilution of synthetic dye between the daughter cells. We find that eGFP is well suitable for this application and can be combined with dextran conjugated to Texas Red for blood vessel visualization.

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Optical Coherence Tomography to Assess Neurodegeneration in Multiple Sclerosis

Axel Petzold

Abstract

Retinal spectral domain optical coherence tomography (OCT) has emerged as a clinical and research tool in multiple sclerosis (MS) and optic neuritis (ON). This chapter summarizes a short OCT protocol as included in international consensus guidelines. The protocol was written for hands-on style such that both clinicians and OCT technicians can make use of it. The protocol is suitable for imaging of the optic nerve head and macular regions as a baseline for follow-up investigations, individual layer segmentation, and diagnostic assessment.

Keywords: Multiple sclerosis, Optic neuritis, Neurodegeneration, Optical coherence tomography

1 Introduction

Time domain optical coherence tomography (OCT) assessment of the retina has made a tremendous impact in the field of multiple sclerosis (MS) (1, 2). State-of-the-art spectral domain OCT now permits for sufficient axial resolution of individual retinal to enable reliable automated layer segmentation. The method stands at the brink of making the transition from a research tool in MS to standard clinical care (3).

There is clear evidence in MS that degeneration affects the inner retinal layers all the way down to the inner nuclear layer (INL) (4). Inner retinal layer atrophy can be seen early in the disease course (5–7). Inner retinal layer atrophy correlates with other structural imaging markers for neurodegeneration such as magnetic resonance imaging (MRI) (8, 6). The causes for correlated atrophy of the inner retinal layer atrophy, the visual pathways, and the primary visual cortex (V1) are anterograde and retrograde *trans*-synaptic axonal degeneration (2, 9, 10). Logically, axonotmesis at any position in the hardwired visual pathways will always result in bidirectional *trans*-synaptic axonal degeneration (11). This concept is illustrated in Fig. 1. In case of the retina, the first-order neuron is the retinal ganglion cell (RGC), the second-order neuron within the dorsal lateral geniculate nucleus (LGN), and the third-order neuron

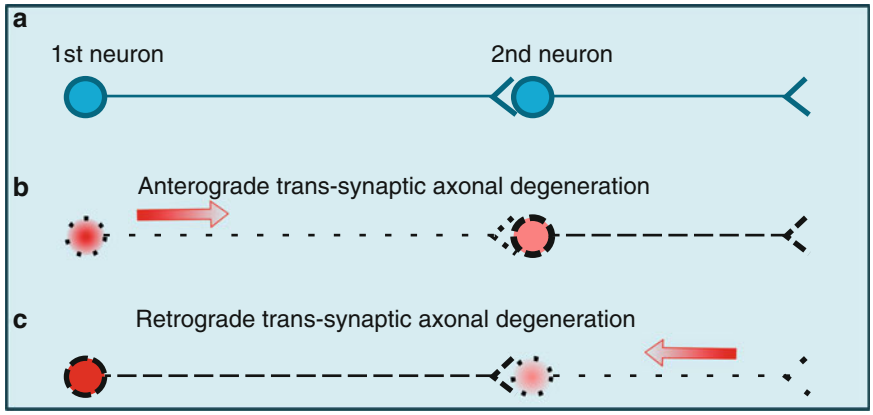

Fig. 1 Bidirectional *trans*-synaptic axonal degeneration in a hardwired pathway. (**a**) Normal situation. (**b**) Axonotmesis of the first axon results in anterograde axonal degeneration (*red arrow* to the *right*). This process can pass on to the second axons *trans*-synaptically. (**c**) Likewise, axonotmesis of the second axon results in retrograde degeneration. Again this process can pass on the first axon *trans*-synaptically. Logically, the process will always be bidirectional

in the visual cortex (V1) (2). In the retina, presence and thickness of retinal layers vary according to location. The most frequently scanned areas by OCT are the peripapillary region (Fig. 2a) and macula (Fig. 2b).

In addition to quantitative OCT data, qualitative assessment has become important (12). Microcystic changes present in the INL have been called microcystic macular oedema (MMO) according to British and microcystic macular edema (MME) according to US spelling (Fig. 3). While these changes are not specific for MS (13, 14), they are of potential prognostic value in MS (15, 16). Therefore, retrograde maculopathy should be the preferred terminology (17). This chapter describes a protocol for retinal OCT of the peripapillary and macular regions useful for the assessment of neurodegeneration in MS.

2 Materials

2.1 Hardware

A retinal OCT device. At the time of writing, this should be a spectral domain OCT device, ideally with an eye tracking function for optimal reproducibility (18, 19). An updated list of the United States Food and Drug Administration (FDA)-approved devices can be found on: www.fda.gov/MedicalDevices. Please consult the manual of your device for specific instructions and safety regulations.

This protocol was written for a Heidelberg Spectralis OCT and Spectralis HRA + OCT. The patient interface for all Spectralis systems is shown in Fig. 4.

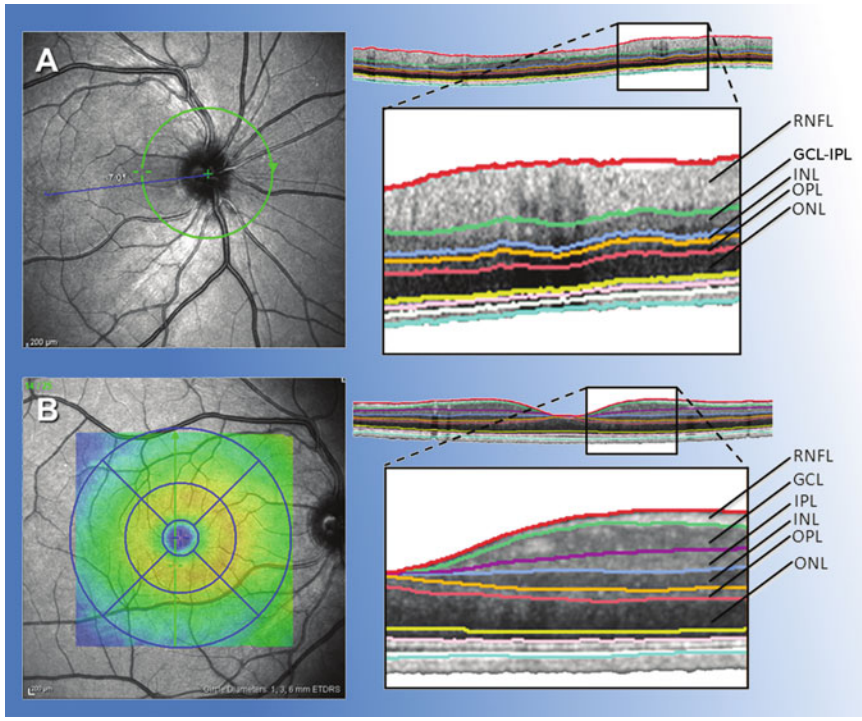

Fig. 2 cSLO images (*left*) and corresponding (*green reference lines*) OCT B-scans (*right*). (**a**) Optic nerve head. Segmented retinal layers are shown for a peripapillary ring scan (*green circle* in Confocal scanning laser ophthalmoscopy (cSLO) image to the *left*). Note the very thin GCL close the optic nerve head explaining why here the combined GCL-IPL (also called ganglion cell complex, GCC) were shown for segmentation. The *blue line* in the cSLO images indicates the physiological tilt of 7° of the foveola with respect to the optic disk. (**b**) Macular region. Segmented layers are shown for a B-scan crossing the foveola (*green vertical line* in cSLO image) constituting the macular volume scan. Note the absence of inner retinal layers from the foveola. *RNFL* retinal nerve fiber layer, *GCL* ganglion cell layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer. Figure reprinted with permission from (28)

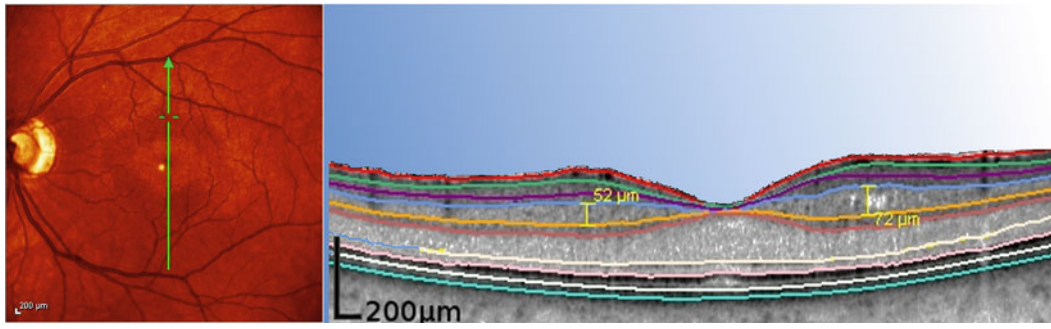

Fig. 3 Microcystic macular edema (MME) of the INL in a patient with RRMS who had experienced optic neuritis in this eye (13). The INL is thickened (72 µm) at site of MME compared to the opposite site (52 µm). The *vertical green line* in the cSLO image (*left*) indicates placement of the OCT B-scan shown to the *right*

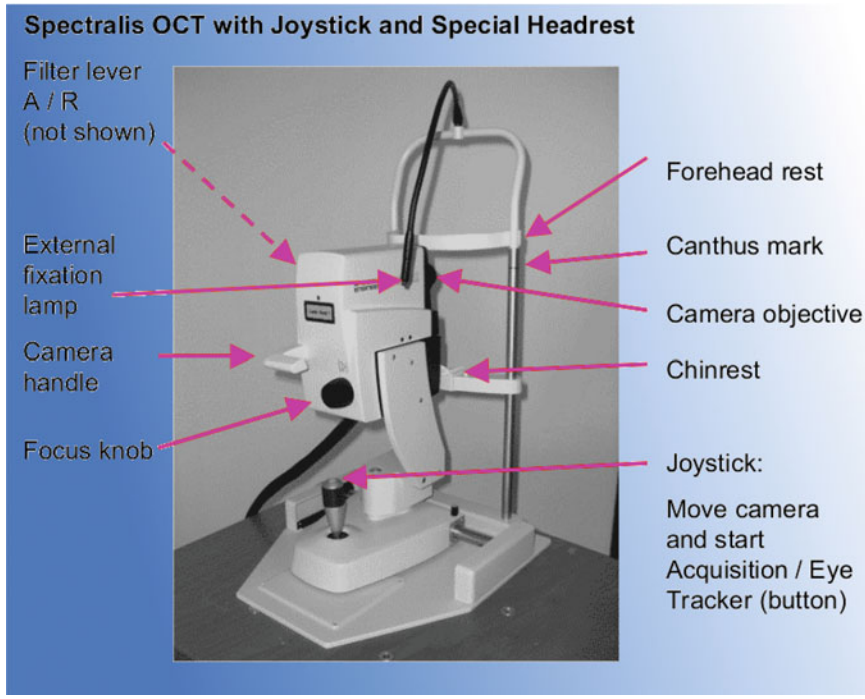

Fig. 4 Photograph of the Heidelberg Spectralis OCT. The *magenta arrows* identify those parts which are discussed in Sect. 2

2.2 Software

The software version at the time of writing is 5.4.8.0 for the acquisition module and 5.4.6.0 for the viewing module. All data are stored in the "Heidelberg Eye Explorer" database version 1.7.0.0. Please take a second to check which software versions your system has. To do so, click on "Help" at the menu bar and then click on "About. . .." The new window will list all software versions. Please note that automated layer segmentation may not be available for older software versions.

3 Methods

3.1 Activating the OCT Device

1. Prepare the device: (a) pull the camera all the way back, (b) clean forehead rest and chin rest, (c) check that the lens is clear, and (d) adjust table height and chin rest so that the canthus marker (*see* Fig. 4) on the right is at the patient's eye height and that the patient feels comfortable.
2. Prepare the patient and inform him/her that there will be a high-pitched noise during the operation. Offer ear plugs in case the patient is phonophobic and cannot tolerate the noise.
3. Start the software, which will open the database on the screen. Create a new patient file. Alternatively open an old patient file if

you have already examined this patient before. Start a new examination. You will be guided to a number of windows allowing you to enter the patient's demographic information. Additional information may be added if required.

4. Select the acquisition mode. You will note that the square on the bottom right of the screen turns yellow. It will take a few seconds before the computer has calibrated and started up the OCT camera. The OCT camera is working when the high-pitched sound starts. The sound is caused by the fast-turning motor that is needed to rotate the mirrors that reflect the light needed for OCT image acquisition. You will see immediately the cSLO image in the large window on the left of the screen. It takes another few seconds before the turning mirror wheel provides enough light information to create an OCT image signal, which will appear in the window on the right side of the screen.
5. Explain to the patient what will happen (*see* Table 1). Next, select a fixation target. There are two options: (a) an internal fixation target is selected by clicking on the point raster (nine points) on the bottom of the screen. The easiest option is to select the point in the middle of the raster by clicking on it. The point will change color from black to blue (on the PC screen), indicating that it is working. The patient sees a blue dot. Some patients may have difficulties seeing the blue internal fixation target either because the vision in the eye to be examined is poor (e.g., optic neuritis) or for other reasons. In this case (b) an external fixation target is needed (*see* Fig. 4). Needless to say

Table 1
Fixation guidelines

Always explain to the patient what he/she will see during the OCT examination during which his/her eye is investigated
The patient will see either a red circle (in the case of pRNFL) or a set of red parallel lines (in the case of the macular scan). As the OCT images are taken, the red light will move. This is particularly noticeable for the red lines. Tell the patient that the eye always reacts sensitively to movement and that this is normal that he/she will eventually look at the moving target. When he/she does so, the OCT measurement will stop (fixation loss) and start again when he/she looks back at the fixation target. Tell the patient that the same will happen if he/she blinks or sits back for a moment. This is not a problem and will only slightly prolong the time of the OCT image acquisition
There are a few patients for whom maintaining fixation will be difficult because of the underlying neurological disease (e.g., nystagmus, oscillopsia). In these patients it is reasonable to turn the automated retina tracking (ART) function off in order to shorten the image acquisition time. Please make a note if you turn ART off. If ART is turned off, this information will automatically be added by the software to the data file

that the patient will look at the external fixation target with the eye that is not being examined by OCT. In some cases a moving fingertip may be easier for a patient than the external fixation lamp. In very few cases with extremely poor vision, an imaginary target will be needed and you will need to instruct the patient to look a tiny bit more to the left/right/up/down.

6. Now ask the patient to sit forward and rest his/her chin on the chin rest and lean his/her forehead against the headrest. Adjust the height of the patient's head such that the canthus of the eye (lateral line between upper and lower lid) is approximately in line with the canthus mark on the metal rod to the right of the camera. Ask the patient if he/she can see the blue light in the camera (internal fixation target). If not, try to provide an alternative internal fixation target or switch to an external fixation target. Good visual fixation is not mandatory but will facilitate and speed-up retinal OCT image acquisition. Encourage patients to blink in between for their own comfort.
7. After selecting the category "Axonal" in the "Application" section, a specific set of "Preset" buttons becomes available. The axonal setting was developed specifically for neurological conditions. The orientation of the OCT B-scans is vertical. This is because all axons projecting from the macular to the optic nerve head will be captured. In contrast the slightly faster horizontally orientated B-scans need to interpolate in between axonal projections with less accurate volumetric information.
8. As a minimal OCT scan protocol, a peripapillary ring scan (Fig. 2a) and a macular volume scan (Fig. 2b) need to be performed. There may be additional preset or custom scan protocols which are beyond the scope of this short protocol.

3.2 Acquisition of the Peripapillary Ring Scan

1. Choose the peripapillary ring scan from the preset buttons. Set resolution and averaging (ART) as wanted. For imaging of MMO an ART of 3–9 may be advantageous. For good contrast images best suited for segmentation, a higher ART is better. There is no golden rule to this. In a patient who can fixate well you will get a suitable image with a low ART. In a patient with poor fixation or opacifications in the media you may need more. Remember the higher the ART, the larger the scanning time. Not all patients may easily tolerate a long scanning time.
2. Activate the "Eye-Tracker" by pressing the button on the joystick for about 2 s.
3. Position the ring scan well centered around the optic nerve head (ONH) using drag and drop with the mouse. Note that a poorly centered ring scan is a rejection criterion for OCT quality control (QC) (20, 21).

4. Define the exact position of the fovea and mark this position (blue line in the SLO image in Fig. 2a).
5. Make sure that the OCT live image gives a good signal and is as horizontally aligned as possible and the chosen number of OCT scan averages (ART) is filled prior to capturing the image with a short click on the joystick button (Fig. 4).

3.3 Acquisition of the Macular Volume Scan

1. Choose a macular volume scan from the preset buttons. Set resolution and averaging (ART) as wanted. When setting ART for a volume scan, please remember that this will take longer compared to the pRNFL scan explained in the previous section.
2. Activate the "Eye-Tracker" by pressing the button on the joystick for about 2 s.
3. Position the volume scan well centered over the foveola using drag and drop with the mouse.
4. Make sure that the OCT live image gives a good signal and is as horizontally aligned as possible and the chosen number of OCT scan averages (ART) is filled prior to capturing the image with a short click on the joystick button (Fig. 4). Always observe the live window to the bottom of the screen to ensure that subsequent B-scans of the volume scan remain of good quality. This will frequently require some minor readjustment of the live image with the joystick during acquisition.

3.4 Post-Acquisition Procedures

Quality control. All retinal OCT scans should be visually inspected during and after acquisition to determine whether the QC criteria will be met (20). These criteria are summarized in Table 2.

Table 2
The OSCAR-IB quality control criteria for retinal OCT scans

Item	Criteria
O	Obvious problems (e.g., unfocused image, wrong scan protocol, etc.)
S	Is the OCT signal sufficient? Signal strength >15, appropriate B-scan averaging (ART function activated)
C	Is the scan correctly centered?
A	Is there an algorithm failure?
R	Is there visible retinal pathology which may potentially impair the RNFL reading? (minor floaters casting a shadow are not relevant)
I	Is the fundus well illuminated?
B	Is the measurement beam placed centrally?

Table modified with permission from reference (20)

Reference scan. Save the images and leave the acquisition window. All SLO images will be displayed as individual image thumbnails. Please only select those images as reference which have passed your own quality control.

If you are happy with QC, mark the thumbnails and set the reference for the "Follow-Up" scan. You can either individually right click on the scans or keep the mouse button pressed and drag a square over all scans together. This allows to set all scans at once with a right click as "Follow-Up."

Automated segmentation. At the time of writing, experience with automated segmentation is undergoing rapid development. A review of the methods available is beyond the scope of this chapter. There are proprietary algorithms which give good results for high-quality images and in absence of any major retinal pathology. It is recommended to always visually revise segmented scans and double-check for algorithm failures.

4 Notes

1. Before starting a scan please make certain (a) that the patient is comfortable and (b) that you have prepared everything you need to have at hand for the scans; (c) this includes having selected and marked baseline scans as reference if you want to acquire follow-up scans.
2. Can the patient fixate? If not, please have a look at the item 5 of the acquisition protocol and Table 1.
3. Remember the most common do's and don'ts (Table 3).
4. Prioritize your needs. Start with the pRNFL scan. This is the quickest scan which already gives a wealth of information. If a patient cannot tolerate longer scanning, you can skip the more time-consuming volume scans.
5. In addition to the scans discussed here, there are scan patterns addressing specific aspects of the retinal anatomy. Each of these has their respective advantages, but will prolong the total imaging time.
6. Remind the patient to blink during the examination if the eyes get dry.
7. Remind the patient to look at the fixation target and not to get distracted by the moving scan lines.
8. Take care that the patient does not speak during the examination. The head will bounce on the chin rest and this will affect the image quality.
9. Remember some patients will have makeup. Always check that the lens is clean prior to scanning.

Table 3
Things to do and things not to do

Do's	Don'ts
Keep the ring scan still while the averaging (ART) is ongoing and only press the button to acquire the image after averaging is complete (horizontal bar under ART)	Do not be trigger active. Premature pressure of the acquisition button will result in low-quality images
Most people are good in getting the first scan well aligned. But please keep an eye on the live imaging window during acquisition of the volume scan. A volume scan is composed of a number of B-scans. Make sure that you do not inadvertently "cut off" subsequent B-scans by either moving the camera too much or not at all	Never ever keep scans which are "flipped over" during acquisition. Repeat them
Keep the scan horizontally aligned in the live image window. If this should not be possible, please make sure that you keep the direction of the tilt on subsequent scans. This is particularly relevant for the ring scans	If there is no chance to get a horizontally aligned scan, remember to tilt it the same way at follow-up

10. Patients with cognitive disabilities, mental disorders (including dementia), movement disorders, or tremor may require you to have an assistant which will hold the head of the patient from the back during scanning.
11. Be systematic but not overly dogmatic. If the composite image looks fine after averaging 40 images and the patient becomes restless, there is no need to wait for averaging of 100 images.
12. Do you have a policy in place in case of accidental findings?
13. If the answer to the above is no, seek advice from your colleagues and/or the ethics committee.
14. Remember that the "penalty" for over-reporting findings you are not entirely sure about is likely to be greater than the that of under-reporting.
15. Do make the safety and comfort of the patient your primary concern throughout the examination and afterward.
16. Under certain conditions you may consider adding extra scans to your protocol. Four examples are discussed below, but there are many more options and selecting the right one will best be guided by common sense.
17. Volume scan of the ONH. This scan gives a more detailed view on the ONH which permits to analyze details around Bruch's membrane (22, 23). In addition, spectral domain OCT permits in some cases to investigate the lamina cibrosa at which point

myelination of the optic nerve starts. For better quality some vendors provide enhanced depth imaging. Future devices may incorporate swept source imaging (24).

18. Volume scan of the papillomacular bundle (PMB). The PMB has long been recognized as a particular vulnerable bundle of retinal nerve fibers (25). Anatomically this is due to the small fiber diameter in the PMB of ≈0.4 μm. The PMB lacks the larger 2.5 μm fibers seen elsewhere in the retina (26). A volume scan of the PMB will permit analyse into greater detail localized atrophy and test related hypotheses on neurodegeneration of this particularly vulnerable structure.
19. Ring scans of increasing eccentricity. Considerable anatomical variability exists for the ONH between individuals. The disadvantage of a single-sized ring scan is that retinal layer thickness will vary considerably between individuals with a small or large ONH. Therefore a set of several ring scans of increasing diameter (as more rapid scan protocol compared to an ONH volume scan) may provide more data, which is likely to be of additional use in the presence of papilledema/pseudo-papilledema (27).
20. Ring scans localized to the opening of Bruch's membrane. The abovementioned interindividual anatomical variations of the ONH make it sometimes difficult to consistently center a ring scan. An elegant solution to this problem is to center the ring scan according to the opening of Bruch's membrane. A semi-automated protocol to this technique does exist for some vendors.

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Part V

Disease Models

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Experimental Autoimmune Encephalomyelitis in Mice

Rachael L. Terry, Igal Ifergan, and Stephen D. Miller

Abstract

Experimental autoimmune encephalitis (EAE), the animal model of multiple sclerosis (MS), has provided significant insight into the mechanisms that initiate and drive autoimmunity. Several central nervous system proteins and peptides have been used to induce disease, in a number of different mouse strains, to model the diverse clinical presentations of MS. In this chapter, we detail the materials and methods used to induce active and adoptive EAE. We focus on disease induction in the SJL/J, C57BL/6, and BALB/c mouse strains, using peptides derived from proteolipid protein, myelin basic protein, and myelin oligodendrocyte glycoprotein. We also include a protocol for the isolation of leukocytes from the spinal cord and brain for flow cytometric analysis.

Keywords: Experimental autoimmune encephalomyelitis, Multiple sclerosis, Autoimmune disease, Mouse model, $CD4^+$ T cells

Abbreviations

CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
IFA	Incomplete Freund's adjuvant
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
PLP	Proteolipid protein
TCR	T cell receptor

1 Introduction

Multiple sclerosis (MS) is a chronic and debilitating autoimmune disease of the central nervous system (CNS), characterized by lesion formation in the white matter of the brain, spinal cord, and optic nerve (1, 2). The precise mechanisms that trigger and drive MS are not completely understood. However, it is clear that myelin antigens are key targets (3). Autoreactive T cells and other immune cells, particularly macrophages, infiltrate the CNS and cause significant damage to the myelin sheath and underlying axons, resulting

in neuronal dysfunction and death (4, 5). Depending on the severity and location of the immune insult, patients may present with a range of neurological symptoms that impact physical functioning (e.g., muscle weakness, numbness or spasms, impaired balance and coordination, fatigue, incontinence) or mental capacity (e.g., memory loss, depression, cognitive difficulties) (6).

The frequency and severity in which MS symptoms occur differs greatly between individuals. Four main categories are used to classify the clinical course of MS in patients (7). Relapsing-remitting MS is the most common clinical pattern observed, and is characterized by recurrent attacks (relapses) followed by periods of remission in which little or no permanent neurological sequelae are evident. Secondary progressive MS describes the clinical course in which patients initially present with relapsing-remitting disease, but develop significant deficits that increase over time. Primary progressive MS describes the clinical course in which patients show progressive worsening of symptoms without relapse or remission phases. Progressive-relapsing MS is characterized by a steadily worsening disease state, in which patients undergo relapses without complete remission (7).

The animal model of MS, Experimental Autoimmune Encephalomyelitis (EAE), aims to replicate the clinical symptoms of disease in vivo, and has been induced in a range of species, including mice, rats, and hamsters (8). Two different methods of EAE induction have been described. Subcutaneous immunization of mice with an emulsion of myelin protein/peptide and complete Freund's adjuvant (CFA) is referred to as "active EAE," and models the induction and effector stages of disease (9). This process results in the direct priming of myelin epitope-specific $CD4^+$ T cells in vivo, which migrate to the CNS and mediate autoimmune responses. In comparison, "adoptive EAE" models the effector phase of disease only. Activated $CD4^+$ T cells are isolated from the draining lymph nodes of immunized mice, restimulated with the initiating myelin protein/peptide in vitro for several days, and then injected into naïve recipients. Disease is typically accelerated, more severe and uniform in comparison to active EAE, with higher incidence (9).

Depending on the initiating protein/peptide, animal species and strain used for the induction of EAE, different clinical etiologies may be mimicked (Table 1). For example, induction of disease in the SJL/J mouse strain using the proteolipid protein (PLP) 139–155 peptide induces relapsing remitting disease (Fig. 1). In comparison, immunization of C57BL/6 mice with the myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide results in a chronic disease course (Fig. 2). Immunization of BALB/c and C57BL/6 mice with the PLP 180–199 peptide also induces chronic disease, but causes relapsing-remitting disease in the SJL/J strain (Fig. 3). The development of transgenic and humanized mice strains, outlined in Table 2, have also proved to be invaluable tools in understanding the

Table 1
Induction of active experimental autoimmune encephalomyelitis in mice

Mouse strain	Myelin peptide/protein	Sequence	Peptide/protein dose (μg)	Pertussis toxin	Clinical course
BALB/c	Whole PLP (10)	From : bovine	200	Yes	Chronic
	$PLP_{180–199}$ (10)	WTTCQSIAFPSKTSASIGSL	200	Yes	
C57BL/6	$MOG_{35–55}$ (11)	MEVGWYRSPFSRVVHLYRNGK	200	Yes	Chronic
	$PLP_{178–191}$ (12)	NTWTTCQSIAFPSK	200	Yes	
	$PLP_{180–199}$[a]	WTTCQSIAFPSKTSASIGSL	200	Yes	
SJL/J	Whole MBP (13)	From: mouse, rat, or bovine	200–400	Yes	Relapsing-remitting
	$MBP_{84–104}$ (14)	VHFFKNIVTPRTPPPSQGKGR	200[b]	Yes	
	$MBP_{89–101}$ (15)	VHFFKNIVTPRTP	200	Yes	
	$MOG_{92–106}$ (16, 17)	DEGGYTCFFRDHSYQ	200[b]	Yes	
	Whole PLP (18)	From: rat or bovine	200	Yes	
	$PLP_{57–70}$ (19)	YEYLINVIHAFQYV	100	Yes	
	$PLP_{104–117}$ (20, 21)	KTTICGKGLSATVT	50	Yes	
	$PLP_{139-151}$ (22)	HSLGKWLGHPDKF	50	No	
	$PLP_{178-191}$ (23)	NTWTTCQSIAFPSK	200	No	
	$PLP_{180-199}$[a]	WTTCQSIAFPSKTSASIGSL	200	Yes	
ABH	Whole MOG (16)	From: rat	200[b]	Yes	Chronic-relapsing
	MOG_{8-22} (16, 24)	PGYPIRALVGDEQED	200[b]	Yes	
	PLP_{56-70} (24)	DYEYLINVIHAFQYV	100[b]	Yes	
PL/J, B10.PL	Whole MBP (25)	From: rat or guinea pig	200	Yes	Chronic/acute monophasic
	MBP_{Ac1-11} (26, 25)	Ac-ASQKRPSQRSK	100	Yes	
	MBP_{35-47} (27)	TGILDSIGRFFSG	200	Yes	
	MOG_{35-55} (28)	MEVGWYRSPFSRVVHLYRNGK	200	Yes	
	PLP_{43-64} (29)	EKLIETYFSKNYQDYEYLINVI	150	Yes	
C3H/HeJ	Whole PLP (18)	From: rat or bovine	200	Yes	Chronic/atypical
	$PLP_{190-209}$ (19, 30)	SKTSASIGSLCADARMYGVL	100	Yes	
	$PLP_{215-232}$ (31, 19)	PGKVCGSNLLSICKTAEFQ	100	Yes	

[a]Terry, Harp, and Miller, unpublished data, Fig. 2
[b]Mice require a second immunization on day 7

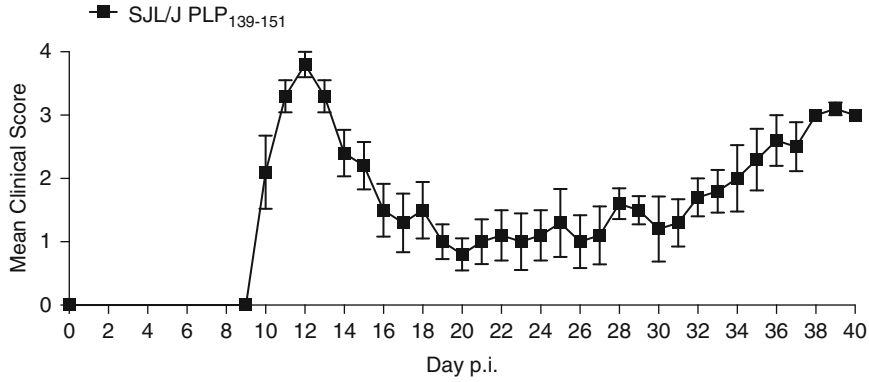

Fig. 1 Induction of EAE in the SJL/J mouse using $PLP_{139–151}$. Immunization of SJL/J mice with 50 μg of the $PLP_{139–151}$ peptide results in a relapsing-remitting course of EAE

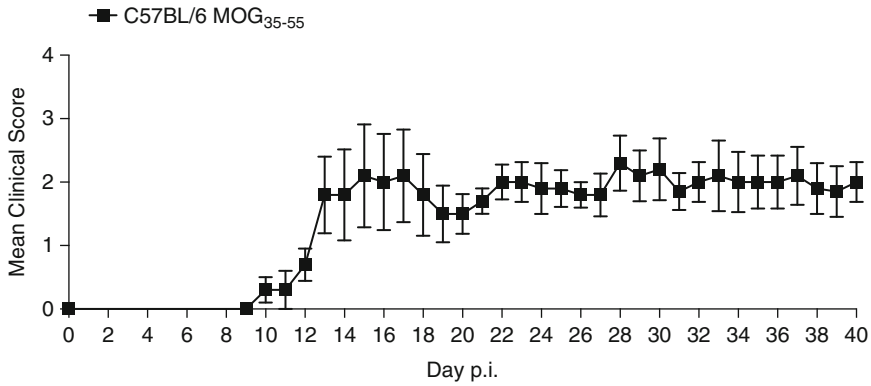

Fig. 2 Induction of EAE in the C57BL/6 mouse using $MOG_{35–55}$. Immunization of C57BL/6 mice with 200 μg of the $MOG_{35–55}$ peptide results in a chronic course of EAE

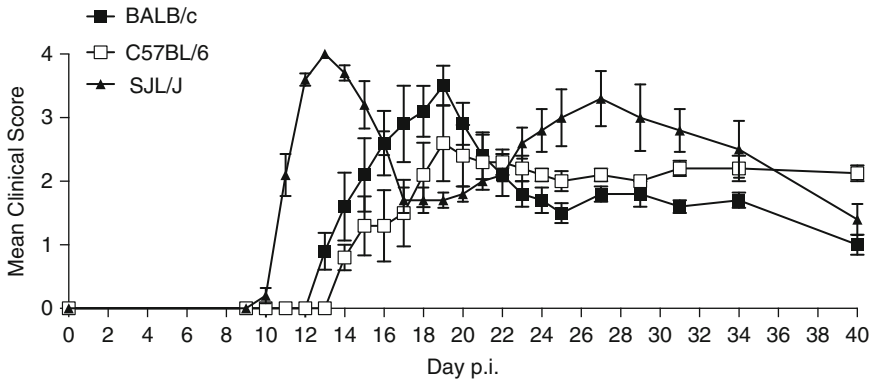

Fig. 3 Induction of EAE in the BALB/c, C57BL/6, and SJL/J mouse using $PLP_{180–199}$. Immunization of BALB/c and C57BL/6 mice with 200 μg of the $PLP_{180–199}$ peptide results in a chronic course of EAE, but causes relapsing-remitting disease in SJL/J mice

Table 2
Transgenic mouse models of experimental autoimmune encephalomyelitis

Background	TCR specificity	Cell-inducing disease	% of spontaneous disease	Age of onset (in weeks)	Clinical course
B10.PL/TCR tg B10.PL/TCR tg × RAG-$1^{-/-}$	MBP_{Ac1-11} (32, 33)	CD4	14–44 % 100 %	5–20 6–20	Chronic/AM Chronic
C57BL/6 HLA-DR2/TCR tg DR2/TCR tg × RAG-$2^{-/-}$	$huMBP_{84-102}$ (34)	CD4	4 % 100 %	ND 7–15	Variable
C57BL/6 HLA-DR15/TCR tg DR15/TCR tg × RAG-$2^{-/-}$	$huMBP_{85-99}$ (35)	CD4	60 % 80–100 %	16–24 5–16	Chronic
SJL/J 5B6	$PLP_{139-151}$ (36)	CD4	40–60 %	6 and older	Chronic
C57BL/6 2D2	MOG_{35-55} (37)	CD4	4–15 % 30–40 %	10–20 10–52	Chronic Optic neuritis
C57BL/6 2D2 × IgH_{MOG}	MOG_{35-55} (38, 39)	CD4 B cells	50–60 %	4–10	Chronic with lesions only in spinal cord and optic nerve
SJL/J TCR1640	MOG_{92-106} (40)	CD4 B cells	60–90 %	8–23	RR on female PP on male
C57BL/6 B7.2 expressed on microglia and T cells	ND (41, 42)	CD8	100 %	12–30	Chronic
C57BL/6 ODC-OVA × OT-I	$OVA_{257-264}$ (43)	CD8	90–100 %	1–3	Chronic/lethal
C57BL/6 HLA-A3/TCR tg	$huPLP_{45-53}$ (44)	CD8	4 %	ND	Motor deficit
NOD 1C6 × IgH_{MOG}	MOG_{35-55} (45)	CD4 CD8 B cells	45–80 %	12–18	RR to chronic

AM acute monophasic, *RR* relapsing-remitting, *PP* primary progressive, *tg* transgenic, *ODC* oligodendrocytes, *ND* not defined

processes that drive autoimmunity. This chapter aims to provide a detailed protocol for inducing EAE in the mouse. Here, we focus on whole proteins and peptides derived from PLP, MOG, and myelin basic protein (MBP). We include the protocols to induce both active and adoptive disease, and the methodology used to isolate single-cell suspensions of leukocytes from brain tissue.

2 Materials

2.1 Active Induction of EAE

1. Female SJL/J, C57BL/6, or BALB/c mice at 6–8 weeks old (Jackson Laboratories).
2. Small animal clippers (e.g., model A5, blade size 50; Oster).
3. Incomplete Freund's Adjuvant (IFA; Difco).
4. *Mycobacterium Tuberculosis* H37Ra, inactivated and desiccated (Difco).
5. Desired myelin protein or peptide (see Table 1).
6. Phosphate-buffered saline (PBS).
7. Pertussis Toxin, if required (see Table 1; List Biologicals).
8. 15 mL polystyrene test tubes.
9. 18 and 25 G needles.
10. 1 mL glass tuberculin syringes with Luer-Lok (VWR).

2.2 Adoptive Induction of EAE

1. Materials listed in Section 2.1.
2. Dissection forceps and scissors.
3. 100 μm sieves (Becton Dickinson).
4. 1 mL syringes.
5. 50 mL conical polystyrene tubes.
6. Recombinant mouse IL-12, if required (R&D Systems).
7. 175 cm^2 culture flasks.
8. 37 °C, 6 % CO_2 tissue culture incubator.
9. 30.5 G needles.
10. Complete Roswell Park Memorial Institute (cRPMI): 440 mL of calcium-free, L-glutamine-free RPMI medium, 5 mL of 100× L-glutamine, 5 mL of 100× Penicillin-Streptomycin, 500 μL of 55 μM 2-mercaptoethanol, and 50 mL of Fetal Bovine Serum (FBS).

2.3 Isolation of CNS Infiltrating Leukocytes

1. Institution-approved anesthetic.
2. Dissection forceps and scissors.
3. 30 mL syringes with Luer-Lok.
4. 21.5 G needles.

5. PBS.
6. Non-treated plastic petri dishes, 60 mm.
7. 5 mL syringes.
8. 18 G needle.
9. Stainless steel wire mesh, 200–300 μm.
10. 15 mL polystyrene tubes.
11. 50 mL conical polystyrene tubes.
12. Liberase, low Thermolysin concentration (Roche).
13. DNase I (Sigma).
14. Percoll (GE Healthcare).
15. 10× no calcium, no magnesium, no phenol red Hank's Balanced Salt Solution (HBSS).
16. 10× no calcium, no magnesium HBSS.
17. 0.5 M EDTA, pH 8.
18. FBS.

3 Methods

3.1 Active Induction of EAE

1. Shave the back of the mice using the small animal clippers (*see* **Note 1**).
2. Prepare complete Freund's adjuvant (CFA) by combining 50 mL IFA and 200 mg *M. tuberculosis* H37Ra, resulting in a final concentration 4 mg/mL *M. tuberculosis* (*see* **Note 2**).
3. Prepare an emulsion of CFA and desired protein/peptide (Table 1) by mixing 1 mL of CFA with 1 mL of desired peptide/protein diluted in PBS. Repeatedly draw up and expel the liquid from a 1 mL glass syringe into a 15 mL polystyrene test tube, using an 18 G needle (*see* **Notes 3–5**).
4. Slowly draw up the emulsion into a new 1 mL glass syringe using an 18 G needle, taking care not to introduce air bubbles. Replace the 18 G needle with a 25 G needle.
5. Inject 100 μL of emulsion subcutaneously into the shaved backs of the mice, distributing evenly over three injection sites. One injection should be placed on the midline of the back just below the shoulders, and two on either side of the midline on the lower back.
6. Refer to Table 1 to determine if pertussis toxin is needed for the mouse strain and initiating protein/peptide you are using. If required, dilute pertussis toxin to 1 μg/mL in sterile PBS (*see* **Note 6**). Inject 200 μL of diluted pertussis toxin (200 ng per mouse) into the peritoneal cavity or intravenously on the day of disease induction, and again 48 h after induction (*see* **Note 7**).

Table 3
Clinical scoring of mice with experimental autoimmune encephalomyelitis

Clinical score	Clinical symptoms
0	Mouse shows no symptoms of disease (asymptomatic)
1	Mouse has a limp tail (complete flaccidity, absence of curling at the tip) or hind limb weakness (waddling gait, mouse's hind limbs fall through the top of a wire cage), not both
2	Mouse has both a limp tail and shows hind limb weakness
3	Mouse has partial paralysis of the hind limbs (can no longer maintain posture of the rump, but can still move one or both limbs to an extent)
4	Mouse shows complete hind limb paralysis (complete loss of movement of the hind limbs, all movement is the result of the mouse dragging on the forelimbs)[a]
5	Moribund (death caused by EAE), mice are euthanized for humane reasons

[a]Mice at this stage of disease are given soft gel food on the cage floor, long sipper tubes, and daily saline injections to prevent dehydration

7. Monitor mice every day to observe the development of clinical symptoms, which usually occur between day 10 and 28 post-induction (see Figs. 1, 2, and 3). Symptoms are measured using the scoring system shown in Table 3.

3.2 Adoptive Induction of EAE

1. Immunize female donor SJL/J, C57BL/6, or BALB/c mice with the desired myelin protein/peptide as detailed in Section 3.1.
2. Prepare cRPMI.
3. Between day 7 and day 14 post-induction (see Table 4), euthanize the mice and remove the draining lymph nodes (inguinal, brachial, and axillary). Place the lymph nodes into cRPMI.
4. Place a 70 μm filter into a non-treated culture plate. Generate a single-cell suspension by pressing the lymph nodes in cRPMI through the filter with the plunger from a 1 mL syringe.
5. Transfer the cRPMI solution containing the cells into a 50 mL polystyrene tube and centrifuge for 10 min at 300 × *g*.
6. Remove the supernatant and raise the pellet in 1 mL of cRPMI and gently resuspend using a pipette. Add another 19 mL of cRPMI to increase the total volume to 20 mL.
7. Count the number of viable white blood cells using trypan blue exclusion of dead cells on a hemocytometer slide.

Table 4
Induction of adoptive experimental autoimmune encephalomyelitis[a]

Mouse strain	Myelin peptide/ protein	Donor immunization period (days)	In vitro peptide/ protein dose (μg/mL)	No. of blasts transferred ($\times 10^6$)	Disease type and severity	Pertussis
BALB/c	$PLP_{180-199}$	10–14	20[b]	5–10	Chronic, Moderate	Yes
C57BL/6	Whole MOG	10–14	50[b]	20	Chronic, Moderate	Yes
	MOG_{35-55}	10–14	10[b]	20	Chronic, Moderate	Yes
	$PLP_{178-191}$	10–14	20	10–20	Chronic, Moderate	Yes
	$PLP_{180-199}$	10–14	20	5–10	Chronic, Moderate	Yes
SJL/J	Whole MBP	7–14	50–100	40–60	RR, Moderate	Yes
	MBP_{84-104}	7–14	50	10–20	RR, Moderate	Yes
	Whole PLP	7–14	50–100	5–10	RR, Severe	Yes
	$PLP_{139-151}$	7–14	20	1–5	RR, Severe	No
	$PLP_{178-191}$	7–14	20	10–20	RR, Severe	No
	$PLP_{180-199}$	10–14	20[b]	5–10	RR, Severe	Yes

RR relapsing-remitting
[a]These parameters have been modified from protocols as previously described for induction of disease in BALB/c (10, 46), C57BL/6 (11, 47–50), and SJL/J (51–58, 20) strains
[b]IL-12 should be added at a dose of 20 ng/mL

8. Culture the cells at a concentration of 8×10^6 cells per mL in cRPMI in 175 cm^2 flasks (up to 30 mL total volume per flask). Add in the required amount of initiating protein/peptide to restimulate the cells (see Table 4; Notes 8–9). Add in 10 ng/mL IL-12 if necessary (see Table 4; Notes 10–11). Incubate for 72 h in a 37 °C, 6 % CO_2 tissue culture incubator (*see* **Note 12**).
9. Harvest the cells by transferring into 50 mL conical tubes and centrifuging at 300 × *g* for 15 min. Count the number of live blasts by using trypan blue exclusion. Blasts will appear larger than other leukocytes in the culture and should comprise at least 10–30 % of the culture.
10. Wash the cells twice in PBS and raise to the required concentration in PBS. Inject the recommended number of blasts in a final volume of 200 μL (see Table 4; **Note 13**) into recipient mice using a 1 mL syringe and 30.5 G needle via intravenous tail vein. Alternatively, blasts may be injected into the peritoneum.
11. Inject 200 μL of diluted pertussis toxin (200 ng per mouse) into the peritoneal cavity or intravenously on the day of disease induction, and again 48 h after induction if necessary (refer to Table 4).

3.3 Isolation of CNS Infiltrating Leukocytes

1. Induce deep anesthesia by injecting mice with approved anesthetic (e.g., Nembutal). Ensure deep anesthesia is achieved by testing reflex responses of the footpads.
2. Draw up PBS into a 30 mL syringe with a 21.5 G needle.
3. Using surgical scissors and forceps, expose the chest cavity by making an incision at the diaphragm and cutting upwards through the rib cage.
4. Using the forceps, carefully hold the heart in place. Make a small incision in the right atrium of the heart.
5. Immediately insert the 30 mL syringe with 21.5 G needle into the left atrium of the heart. To ensure correct placement, put a small amount of pressure on the syringe plunger. An efflux of dark red blood should immediately flow from the right atrium (*see* **Note 14**). Slowly perfuse the animal by continuing to apply pressure on the plunger (*see* **Notes 15**, **16**).
6. Remove the head of the mouse by carefully cutting under the skull, through the neck. Using scissors or a scalpel, cut the skin and cutaneous muscle by making a single incision along the midline length of the head, from the posterior aspect of the skull to the nose of the mouse. Using the forceps, fold back the two flaps of skin to expose the skull.
7. Carefully cut through the top of the skull along the midline, from the posterior aspect of the skull to the nose. Make two incisions from the posterior aspect of the skull to the eye sockets, and use the forceps to remove the pieces of skull from the top of the head. Carefully remove the brain from the cranial cavity and place in a 50 mL tube containing 20 mL of PBS.
8. Using a scalpel or scissors, make a long incision through the midline of the mouse (from the neck to the base of the tail). Peel the skin back to expose the spinal column. Identify where the base of the spinal column attaches to the pelvis, and make a perpendicular cut through the spine at this point. Cut along each side of the column to the neck to remove the column.
9. Attach an 18 G needle to a 5 mL syringe that has been filled with PBS. Hold the column in a petri dish with forceps, and insert the needle into the spinal column at the caudal end. Push the plunger of the syringe to expel PBS into the spinal column. Initial resistance should be felt, followed by release, in which the spinal cord will be flushed from the column. Transfer spinal cord into a 50 mL tube containing 20 mL PBS.
10. Carefully pass the brain and spinal cord through the stainless steel wire mesh in the 60 mm petri dishes using the plungers taken from 10 mL syringes. Rinse all tissue off the mesh with additional PBS. Transfer back into 50 mL tubes and centrifuge for 20 min at 300 $\times$ *g* at 4 °C. Remove the supernatant.

11. Prepare digestion enzyme mix by adding 1 g DNase I (50 μg/mL final concentration) and 800 U Liberase (40 U/mL final concentration) to 20 mL PBS (*see* **Note 17**). Raise cells in 2 mL of digestion enzyme mix and incubate for 30 min at 37 °C.
12. Prepare 500 mL FACS buffer by adding 10 mL FCS (2 % final concentration) and 2 mL 0.5 M EDTA (2 mM final concentration) to 488 mL PBS.
13. Add 30 mL FACS buffer to samples and pass through a 70 μM sieve into a new 50 mL tube. Centrifuge for 10 min at 300 × *g* at 4 °C. Wash cells again twice in FACS buffer.
14. Prepare 30 % Percoll by adding 5 mL 10 % HBSS (with phenol red) and 15 mL Percoll to 35 mL of water. Prepare 70 % Percoll by adding 5 mL 10 % HBSS (without phenol red) and 35 mL Percoll to 15 mL of water (*see* **Note 18**).
15. Raise the CNS samples in 5 mL of 30 % Percoll and transfer to a 15 mL tube. Underlay samples with 5 mL of 70 % Percoll. Centrifuge the samples at 1,000 × *g* for 25 min at room temperature with no brake.
16. Using a pipette, remove the myelin layer from the top of the gradient. Using a new pipette tip, collect the mononuclear cells at the interface between the 30 and 70 % Percoll interface and transfer into a new 15 mL tube containing 5 mL FACS buffer. Centrifuge for 10 min at 300 × *g* at 4 °C. Wash cells again twice in FACS buffer.
17. Count live cells using trypan blue exclusion.
18. Single cells can now be further processed for the desired assay, e.g., flow cytometric analysis.

4 Notes

1. It is recommended to shave the back of the mice at least 24 h before inducing disease. The animals will then be easier to handle on the day of induction.
2. It is critical to purchase IFA and *M. tuberculosis* separately and prepare CFA at a final concentration of 4 mg/mL. Commercially available CFA only contains 1 mg/mL *M. tuberculosis*.
3. The lowest concentration of a given myelin protein/peptide that can be used to reliably induce EAE may vary by source and batch number. The source and age of the mice used may also alter the disease course from that expected. Thus, the concentrations of protein/peptide indicated in Table 1 should be used as a guide only and should be confirmed by the individual investigator before conducting large-scale experiments.

4. CFA can be prepared up to 24 h in advance and stored it in polystyrene tubes or glass syringes at 4 °C until use.
5. To test the consistency of the emulsion, a small droplet should be expelled onto the surface of water in beaker. If the emulsion is stable, the droplet will remain in a bead on the water surface. If the droplet disperses across surface, further emulsification is required.
6. It is recommended to dissolve the lyophilized pertussis toxin in sterile PBS at least 24 h before injection and store at 4 °C until use.
7. We inject 200 ng of pertussis per mouse on day 0 and day 2 post-immunization where indicated on Table 2. However, some papers report injection of up to 500 ng of pertussis per mouse. The individual investigator should determine the optimal dose for the protein/peptide and mouse strain used.
8. The amount of peptide needed to effectively restimulate T cells in vitro may vary by protein/peptide source and batch number. The source and age of the mice used may also affect the amount of protein/peptide needed for effective restimulation. Thus, the concentrations of protein/peptide indicated in Table 4 should be used as a guide only and should be confirmed by the individual investigator before conducting large-scale experiments.
9. Con A may be used at a dose of 1 μg/mL to stimulate T cells for 48 h in vitro in place of specific myelin protein/peptide for the induction of adoptive EAE. Con A activation, however, will reduce the frequency of myelin epitope-specific T cells in the culture, thus an increased number of total cells will need to be injected into recipient mice. The individual investigator will need to titrate these numbers in vivo to determine the lowest number of cells required to achieve severe and reliable EAE.
10. In addition to IL-12, IL-2 may also be added into the media at a concentration of 10 ng/mL to enhance T cell activation and proliferation. The individual investigator should confirm the optimum concentration of these cytokines before conducting large-scale experiments.
11. IL-23 may be added to the media at a concentration of 10 ng/mL to induce a Th17-skewed T cell phenotype. The individual investigator should confirm the optimum concentration of IL-23 before conducting large-scale experiments.
12. We typically incubate cells in vitro for 3 days before transferring into recipients. Most papers report incubation periods of 3–4 days. The individual investigator should determine the optimal incubation time for the protein/peptide and mouse strain used.

13. The lowest number of cells that are needed to reliably induce EAE may vary by myelin protein/peptide source and batch number. The source and age of the mice used may also alter the disease course from that expected. The numbers listed in Table 4 should be used as a guide only. The individual investigator will need to titrate these numbers in vivo to determine the lowest number of cells required to achieve severe and reliable EAE.
14. If the needle is not placed correctly, dark red blood will not flow from the right atrium and the lungs may inflate. Loss of red coloration of the liver is a good indicator of correct perfusion. The authors recommend practicing this technique before conducting large-scale experiments.
15. Perfusions should be conducted slowly (over a period of at least 3–5 min per mouse) to avoid tissue damage.
16. If the mouse is not well perfused after the initial procedure, the syringe may be refilled with 30 mL of PBS and perfusion repeated.
17. The protocol listed here using Liberase and DNase is optimized for the isolation of total leukocytes. Different enzymatic digestion may be performed on the CNS tissues to isolate different target cell populations. For example, we have found that digesting the CNS using Accutase (Millipore) in place of Liberase and DNase is optimal for the isolation of oligodendrocyte progenitor cell isolation.
18. The use of HBSS with and without phenol red for the 30 % Percoll and 70 % Percoll solutions, respectively, will increase the ease of which to see the interface between the two gradients and identify the mononuclear cell layer here.

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Actively Induced Experimental Autoimmune Encephalomyelitis in Rats

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Abstract

The rat and especially a number of inbred rat strains are very well suited for modeling multiple sclerosis (MS). Experimental autoimmune encephalomyelitis (EAE), the model of MS, can be induced by active or passive immunization. Active immunization can be performed with different myelin proteins or peptides thereof. Passive immunization is performed by transfer of myelin-specific T cells. Most known is EAE induced with myelin basic protein (MBP) in LEW ($RT1^{l}$) rats that results in monophasic disease and EAE induced with myelin oligodendrocyte glycoprotein (MOG) in DA ($RT1^{av1}$) rats that leads to relapsing remitting or chronic disease. Depending on the selected inbred rat strain, the immunogen and adjuvant used, different disease courses and pathologies can be induced that mimic different aspects of MS.

Keywords: Rat, Inbred, Experimental autoimmune encephalomyelitis, Disease course, Monophasic, Relapsing-remitting, Chronic, Neuromyelitis optica, Optic neuritis

1 Introduction

Multiple sclerosis (MS) follows a relapsing remitting or chronic disease course (1). The disease can be active or inactive (2, 3). Activity is mainly related to inflammation of the central nervous system (CNS) in regard to infiltration with lymphocytes and presence of contrast-enhancing lesions. In the past MS has been mainly considered as a disease of the white matter. More recently there is also much evidence for presence of grey matter affection with cortical pathology in MS (4). Based on the heterogeneity in disease courses and lesion pathology, it is difficult to model all aspects of MS in one animal model. Therefore the availability of different inbred and outbred rat strains (Table 1) and different immunogens and adjuvants is very valuable since it allows inducing different types of diseases and pathologies. Most useful are inbred rat strain and inbred congenic rat strains, which differ in certain genomic regions and allow modeling of the influence of the genomic region on disease course and pathology in EAE. We have used this approach in the past and established various models of MS that mimic different disease courses and pathologies in LEW congenic inbred rat strains (5, 6) (Tables 2 and 3). Besides the rat strain used,

Table 1
RT1 haplotypes of inbred rat strains (5)

Strain[a]	Haplotype	Class I		Class II	Class III	Class I
		RT1.A	RT1.B	RT1.D		RT1.C
DA	av1	a	a	a	av1	av1
COP	av1	a	a	a	av1	av1
ACI	av1	a	a	a	av1	av1
PVG-RT1AV1 (DA)	av1	a	a	a	av1	av1
LEW.1AV1 (DA)	av1	a	a	a	av1	av1
LEW	l	l	l	l	l	l
LEW.1N (BN)	n	n	n	n	n	n
LEW.1A (AVN)	a	a	a	a	a	a
LEW.1W (WP)	u	u	u	u	u	u
LEW.1AR1	r2	a	u	u	u	u
LEW.1AR2	r3	a	a	a	u	u
LEW.1WR1	r4	u	u	u	a	a
LEW.1WR2	r6	u	a	a	a	a
BN	n	n	n	n	n	n

RT1.B is the rat equivalent to HLA-DQ and RT1.D to HLA-DR
[a]Donor strain in brackets

Table 2
Presently most used EAE models for laboratory research

Species	Strain	Induction	Disease type	Reference
Rat	LEW	MBP or MBP 68–88 peptide in CFA	Acute monophasic	(11, 12)
Rat	DA	Spinal cord in IFA or MOG 1–125 in CFA or IFA	Relapsing/progressive	(5, 13)

The list only provides a part of all available EAE models
CFA complete Freund's adjuvant, *IFA* incomplete Freund's adjuvant

the immunogen and the adjuvants are also of great importance. Active immunization of LEW ($RT1^l$) rats with myelin basic protein (MBP) results in monophasic EAE (7) (Tables 2 and 3). There is mainly infiltration of T cells in the CNS and presence of perivenous inflammation. This model has been widely used in the past in many ground-breaking publications regarding basic immunological principles in EAE and MS. We introduced active immunization with myelin oligodendrocyte glycoprotein (MOG) in DA ($RT1^{av1}$) rats,

Table 3
Immunization regimen for different EAE models and usage of adjuvants

Strain	Immunogen	Primary adjuvant	Secondary adjuvant	Injection site	Reference
LEW	MBP	CFA	None	Tail base	(14)
LEW	MBP 68–88	CFA	None	Tail base	(5)
LEW	MBP 89–101	CFA	None	Tail base	(14)
LEW	PLP	CFA	None	Tail base	(15)
DA, LEW.1AV1, BN, LEW.1N, LEW1.AR1	MOG 1–125	CFA or IFA	None	Tail base	(5, 8, 16)
DA, LEW.1AV1	MOG 91–108, MOG 96–104	CFA	None	Tail base	(9, 10)
LEW.1N or BN	MOG 91–108, MOG 98–106	CFA	None	Tail base	(9, 10)

CFA complete Freund's adjuvant, *IFA* incomplete Freund's adjuvant

Table 4
EAE models induced with MOG 1–125 in rats

Species	Strain	Induction	Disease type	Reference
Rat	LEW.1A	MOG 1–125 in CFA	Chronic progressive	(5)
Rat	LEW.1AV1	MOG 1–125 in CFA	Relapsing-remitting	(5)
Rat	LEW.1AV1	MOG 1–125 in IFA	Relapsing-remitting	(16)
Rat	LEW.1N	MOG 1–125 in CFA	Hyperacute progressive	(5)
Rat	LEW.1N	MOG 1–125 in IFA	Chronic progressive	(16)
Rat	LEW.1W	MOG 1–125 in CFA	Slow progressive	(5)
Rat	LEW.1AR1	MOG 1–125 in CFA	Slow progressive, cortical pathology	(5, 8)
Rat	DA	MOG 1–125 in CFA	Relapsing-remitting, optic neuritis	(5, 6)
Rat	BN	MOG 1–125 in CFA	Neuromyelitis optica	(5, 17)

Different disease courses and types of CNS pathology can be induced that are dependent on the MHC haplotype

BN ($RT1^n$) rats, and inbred congenic LEW rats to induce relapsing-remitting and chronic disease courses as well optic neuritis and neuromyelitis optica (5, 6) (Tables 3, 4, and 5). In addition we were able to model cortical lesions in EAE in selecting LEW.1AR1 ($RT1^{r2}$) rats for disease induction (8) (Tables 3 and 4). By doing this we have enlarged the spectrum of EAE models that can model specific aspects of MS.

Table 5
EAE models induced with MOG peptides

Species	Strain	Induction	Disease type	Reference
Rat	DA	MOG 91–108 in CFA	Monophasic or chronic	(9)
Rat	LEW.1AV1	MOG 91–108 in CFA	Monophasic or chronic	(9)
Rat	LEW.1N	MOG 91–108 in CFA	Monophasic or chronic	(9)
Rat	LEW.1AV1	MOG 96–104 in CFA	Monophasic or chronic	(10)
Rat	LEW.1N	MOG 98–106 in CFA	Monophasic or chronic	(10)

Based on detailed immunological analysis, the region MOG 91–108 was defined as the encephalitogenic region in different rat strains

2 Materials

All materials and reagents should be sterile. Also all material should be endotoxin free.

1. 1× Phosphate Buffered Saline (PBS)
2. 70 % alcohol
3. Sterile 50 ml plastic tubes
4. Sterile 12 ml plastic tubes
5. Sterile plastic pipettes
6. Sterile syringes, Luer lock, 10 ml
7. Sterile syringes, Luer lock, 5 ml
8. Sterile syringes, Luer lock, fine dosing, 1 ml
9. Sterile needles, ø 1.2 × 40 mm
10. Sterile needles, ø 0.60 × 30 mm
11. Sterile three way cock
12. Combi stopper
13. Freund's adjuvant, incomplete (Sigma)
14. *M. Tuberculosis* H37 Ra, desiccated, 100 mg (Becton, Dickinson and Company)
15. Immunogen (recombinant proteins, peptides) (Table 6)
16. Diethyl ether (store in a vented flammable material cabinet)
17. Inbred rats (Table 1)

3 Methods

Take care that everything is done in a sterile way and with sterile material.

Table 6
Sequences of myelin peptides used for EAE induction in rats

Myelin protein stretch	Sequence	Reference
MBP_{RAT} 68–88	HYGSLPQKSQRTQDENPVVHF	(7)
MBP_{GP} 68–88	HYGSLPQKSQRSQDENPVVHF	(7)
MBP_{RAT} 89–101	VHFFKNIVTPRTP	(7)
MOG_{RAT} 91–108	SDEGGYTCFFRDHSYQEE	(9)
MOG_{RAT}Ac 96–104	Ac-YTCFFRDHS-NH2	(10)
MOG_{RAT}Ac 98–106	Ac-CFFRDHSYQ-NH2	(10)

3.1 Preparation of Immunization Emulsion

1. Decide on the immunization type which will be used: active immunization with complete Freund's adjuvant or active immunization with incomplete Freund's adjuvant (*see* **Note 1**).
2. Per rat prepare 100 µl of complete or incomplete Freund's adjuvant (ICA). If you use complete Freund's adjuvant (CFA), normally use 200 µg of desiccated *M. Tuberculosis* H37 Ra (MT) per rat (*see* **Note 2**). For preparation of complete Freund's adjuvant, add 200 µg of MT to 100 ml of ICA.
3. Dilute the immunogen with a concentration of 100 µg per rat for peptides or 50 µg per rat for recombinant proteins in 100 µl PBS (*see* **Note 3**).
4. Mix the complete or incomplete adjuvant with the immunogen in PBS 1:1 in a 12 or 50 ml plastic tube (*see* **Note 4**). Initially, use a vortex to mix in order to obtain a pre-emulsion.
5. Depending on the total volume of the pre-emulsion, use a 1, 5, or 10 ml syringe and collect the inoculum in the syringe with a ø 1.2 × 40 mm needle (*see* **Note 5**).
6. Connect the syringe with the inoculum with a three way cock with an empty syringe of similar size and carefully move the inoculum from one syringe to the other one. Continue to do this for several minutes until you obtain a homogenous emulsion (*see* **Note 6**).
7. Transfer the inoculum in a plastic tube and store at 4 °C until use (*see* **Note 7**).
8. Before immunization, collect the emulsion in sterile 1 ml fine dosing syringes. Try to avoid air bubbles in the syringe (*see* **Note 8**).

3.2 Anesthesia of Rats

1. Put some drops of diethyl ether on gauze in an inhalation chamber. If the use of ether is not allowed in your animal facility, use an alternative that is recommended by the veterinarian of your animal facility (*see* **Note 9**).
2. Put the rat into the chamber until it falls asleep.
3. Take the rat out of the chamber for immunization.

3.3 Immunization of Rats

1. Disinfect the rat at the base of the tail with alcohol (*see* **Note 10**).
2. Inject 200 μl of the emulsion intradermally or subcutaneously at the base of the tail. Use a ø 0.60 × 30 mm needle for immunization (*see* **Note 11**).

3.4 Freezing of Inoculum

1. The emulsion can be frozen at −20 °C and thawed just before use (*see* **Note 12**).

3.5 Scoring of Rats

1. The rats should be weighed and scored on a daily basis (*see* **Note 13**).
2. The signs of disease are scored as follows: Grade 1, tail weakness or tail paralysis; Grade 2, hind leg paraparesis or hemiparesis; Grade 3, hind leg paralysis or hemiparalysis; Grade 4, complete paralysis (tetraplegy); Grade 5, moribund state or death (*see* **Note 14**).
3. The rats with MBP-induced monophasic EAE should be scored for 25 days; the rats with relapsing-remitting or chronic EAE should be scored for 40 days or longer (*see* **Note 15**).

4 Notes

1. First, it is necessary to decide clearly on the type of research question. Depending on the question which is asked the model should be selected. EAE induced with MBP in LEW ($RT1^{l}$) rats is a good model to assess basic immunological mechanisms in regard to T cell function in autoimmune disease. EAE induced with MOG in DA rats ($RT1^{av1}$) is better suited to assess immune mechanisms relevant to MS. T and B cells as well as macrophages are recruited to the lesion site. This results in demyelination and axonal loss. Treatment strategies that can halt or alter the disease course in MS can be assessed in this model.
2. Count the number of rats you want to immunize. Multiply the indicated number of rats with the amount of IFA and MT. Add 2–3 additional immunizations to compensate loss of emulsion during preparation. Prepare the calculated amount.

3. As above multiply the indicated number of rats with the amount of immunogen you will use. Add for safety 2–3 additional immunization. Prepare the calculated amount.
4. Depending on the total volume, use a 12 or 50 ml plastic tube. For several minutes, mix with a vortex the plastic tube with the pre-emulsion until you obtain one single phase.
5. Carefully draw up all the pre-emulsion in the syringe with the large diameter needle (ø 1.2 × 40 mm). Try not to get air bubbles into the syringe.
6. The emulsion is ready when a drop of it floats on water.
7. Mix with a vortex the emulsion before you use it or repeat the procedure with the two syringes if the emulsion is not homogenous any more. Do not leave for more than 6 h at 4 °C. If you need more time, freeze the emulsion at −20 °C.
8. To draw up the emulsion into the fine dosing 1 ml syringes that are used for immunization, use a large diameter needle (ø 1.2 × 40 mm). Avoid air bubbles. The fine dosing syringe should have marks which allow you to dose exactly 200 µl at immunization. For immunization use a needle with a small to medium diameter (ø 0.60 × 30 mm).
9. Please discuss the anesthesiology in detail with the veterinarian at your animal facility and also describe the immunization procedure in detail in the application for the regional board in order to obtain permission to perform animal experimentation.
10. The immunization at the base of the tail is superior to food-pad immunization. In the past, most immunizations in rats were done by food-pad immunization. This can result in massive swelling of the feet. Firstly, this is very painful for the animal and must be prevented. Secondly, the massive swelling can mask EAE symptoms. The immunization at the base of the tail can result in a slight swelling but, if performed well, does not affect the rat. The tail-base immunization can be done in a very reproducible way.
11. Before you go to the animal facility for the immunizations, distribute the emulsion in the fine dosing syringes that you use for the immunization and put these on ice.
12. It is possible to freeze and thaw the emulsion. The freezing and thawing cycles should be reduced to a minimum. At each freeze-thaw cycle, the emulsion can slightly change its physico-chemical properties, which can result in a different immunogenic potential. Therefore it is recommended to either not freeze at all or aliquot before freezing so each immunization can be performed with a similar type of emulsion. After thawing, the emulsion needs to be mixed again with a vortex and emulsified in two syringes connected by a three way cock by moving it backwards and forwards.

13. Weighing of the rats allows you to monitor for early signs of disease and is an additional parameter of disease.
14. Depending on the model used, the rats will develop certain symptoms. Sometimes disease phenotype can be atypical. Possibly alternative scoring schemes should be selected. Rats with optic neuritis often wipe the affected eye(s).
15. In monophasic EAE induced with MBP in LEW ($RT1^{l}$) rats, disease onset is normally seen at day 8–9 postimmunization (p.i.). In DA ($RT1^{av1}$) rats immunized with MOG, disease onset is at about 12–14 days p.i. Depending on the rat strains, disease onset can be as late as 25–30 days p.i.

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Experimental Autoimmune Encephalomyelitis in Marmosets

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Abstract

Experimental autoimmune encephalomyelitis (EAE) in the common marmoset, a small-bodied Neotropical primate, is a well-known and validated animal model for multiple sclerosis (MS). This model can be used for exploratory research, i.e., investigating the pathogenic mechanisms involved in MS, and applied research, testing the efficacy of new potential drugs.

In this chapter, we will describe a method to induce EAE in the marmoset. In addition, we will explain the most common immunological techniques involved in the marmoset EAE research, namely isolation of mononuclear cells (MNC) from peripheral blood and lymphoid tissue, assaying T cell proliferation by thymidine incorporation, MNC phenotyping by flow cytometry, antibody measurement by ELISA, generation of B cell lines and antigen-specific T cell lines, and assaying cytotoxic T cells.

Keywords: Callithrix jacchus, Marmoset, Experimental autoimmune encephalomyelitis (EAE), Multiple sclerosis, Myelin oligodendrocyte glycoprotein (MOG), Immune profiling, Flow cytometry, Mononuclear cells, Cytotoxicity, T cell proliferation

1 Introduction

Experimental autoimmune encephalomyelitis (EAE) in the common marmoset is a well-known and validated animal model for multiple sclerosis (MS). This model can be used for exploratory research, i.e., investigating the pathogenic mechanisms involved in MS, and applied research, testing the efficacy of new potential drugs. Successful intervention of the disease with new drugs will also lead to more insight into the pathogenic mechanisms. Human therapies based on biological molecules, monoclonal antibodies, or (antagonists of) cytokines are highly species-specific and therefore often precludes safety and efficacy testing in rodent models, thus creating a need for disease models more closely related to humans [1].

The common marmoset (*Callithrix jacchus*) is a New World monkey originating from the northeastern regions of Brazil. Marmosets have an average lifespan of 10 years, but this can be 15–20 years in captivity. In their natural habitat, both sexes are of similar size; i.e., approximately 20 cm in length, with a tail of 30 cm,

and a body weight ranging from 250 to 350 g. Sexual maturity is reached at 18 months. Mothers give birth to two twins or triplets per year [2].

The common marmoset offers several practical advantages. First, the small body size of the adult marmoset requires much less test compound than needed for an adult macaque (4–10 kg) and also housing and handling are therefore easier. Second, marmosets are born as bone-marrow chimeric twins or triplets due to fusion of the placental bloodstream in utero. These immunological comparable twins provide an ideal setting for placebo-controlled efficacy evaluation of a new treatment [3, 4]. Third, their nonaggressive nature enables handling for routine procedures without the need for sedation. Fourth, a major advantage of marmosets, in contrast to specific pathogen-free rodents, is that they are exposed to several immune shaping pathogens such as the marmoset counterparts of cytomegalovirus and Epstein-Barr virus (EBV). Finally, their outbred nature makes them more comparable to the human genetic variation.

On the other side, there are some challenges in immunology research in marmosets, including the high costs of the animals and ethical constraints. Furthermore, the small body size limits the collection of blood for immune profiling to about 3.5 ml per month (= ±1 % of the monkey's body weight). Another hurdle is the limited availability of validated reagents for immunophenotyping by flow cytometry, the detection of antibody or cytokine levels in serum or cell culture supernatants by ELISA, and for immunohistochemical analysis of tissues.

In this chapter, we will describe a method to induce EAE in the marmoset and the most common immunological techniques involved in the marmoset EAE research, namely isolation of mononuclear cells (MNC) from peripheral blood and lymphoid tissue, assaying T cell proliferation by thymidine incorporation, MNC phenotyping by flow cytometry, antibody measurement by ELISA, generation of B cell lines and antigen-specific T cell lines, and assaying cytotoxic T cells.

EAE in the marmoset can be induced by using myelin, myelin proteins, or myelin peptides [5, 6]. The most well-established immunization protocol is by intradermal injection of recombinant human myelin oligodendrocyte glycoprotein (rhMOG1-125) or with the synthetic human myelin oligodendrocyte glycoprotein peptide corresponding to amino acid 34–56 (MOG34-56) emulsified in incomplete Freund's adjuvant (IFA) or complete Freund's adjuvant (CFA) [7]. Depending on the research question the appropiate antigen and adjuvant is chosen for immunization. For example, the disease progression is mainly T cell driven when the animals are immunized with MOG34-56 in IFA, while immunization with rhMOG in CFA leads to a more B cell driven disease.

After termination of an EAE experiment, mononuclear cells (MNC) can be isolated from blood (PBMC) or secondary

lymphoid organs to study the immuno-pathogenesis. The secondary lymphoid organs isolated are usually the spleen, axillary (ALN), inguinal (ILN), lumbar (LLN), and cervical (CLN) lymph nodes, but other lymph nodes can be removed and processed as well. The advantage of blood-derived MNC is that these can be collected before the start of the study to determine baseline levels of certain immunological parameters. In addition, blood can also be collected during the whole experiment to monitor changes, for example depletion or increase of certain cell subsets. MNC are usually used for T cell profiling assays and to determine the phenotype of cells.

The most commonly used assay to test reactivity of MNC against several antigens, for example against MOG34-56 in rhMOG or MOG34-56 immunized marmosets, is the T cell proliferation assay. There are several methods available to assess proliferation. This chapter will describe the thymidine incorporation assay. In this assay a radioactive labeled nucleoside, e.g., ^{3}[H]-thymidine, is added to antigen-stimulated MNC, after which it is incorporated into any newly synthetized DNA strand during mitotic cell division. In response to antigen exposure, antigen-specific T cells present in the cell culture start to proliferate and therefore incorporate more ^{3}[H]-thymidine than unstimulated cells. Afterwards, the amount of incorporated radioactivity in the DNA is measured by a beta-counter to determine the size of the proliferative response. The advantage of using thymidine incorporation assay for the marmoset is that low cell numbers (100,000–200,000 cells/well) can be used to obtain reliable data.

To get more insight into the phenotype of the cells that are involved in the disease, MNC can be phenotyped by using commercially available monoclonal antibodies (mAb) for flow cytometry. Since most mAb are raised against humans, mice, and rhesus macaques, cross reactivity tests have been performed with these mAb and marmoset cells [8]. We have already performed such cross reactivity assays for a large panel of mAb. The most commonly used antibodies with their corresponding clone number are listed in Table 1.

Another important immunological marker in an EAE experiment is the humoral activity. Therefore, the antibody profile is usually determined by ELISA (enzyme linked immuno-sorbent assay), a biochemical assay to determine whether a certain antigen is present in a sample. The method that we use to determine marmoset antibody levels is highly similar to other protocols, although some small modifications were made to test antibody responses against as much antigens as possible. Plasma obtained from repeated blood draws during the study gives us the opportunity to monitor longitudinal antibody responses.

Since several methods have already been mentioned to profile the marmoset immune response ex vivo, we would also like to draw attention to a more peptide-specific cellular experiment, such as a

Table 1
Monoclonal antibodies that are cross reactive with marmoset cells

Antibody	Clone	Provider*
CD1d	LY38	1
CD3	SP34-2	1
CD4	L200	1
CD5	UCHT2	3
CD8	LT8	4
CD9	ML13	1
CD11b	D12	1
CD11c	S-HCL-3	1
CD16	3G8	1
CD20	H299	5
CD21	HB5	3
CD23	ML233	1
CD25	2A3	1
CD27	MT271	1
CD39	eBioA1	3
CD40	B-B20	6
CD45RA	5H9	1
CD56	Ncam 16-2	1
CD80	L307.4	1
CD83	HB15a	5
CD86	IT2.2	1
CD95	DX2	1
CD127	eBioRDR5	3
CD355	BAB281	5
CD278	isa3	3
CD279	J105	3
CCR4	205410	7
CCR7	150503	7
Foxp3	PCH101	3
HLA-DR	L243	1

*Provider: *1* BD Biosciences, *2* Dako, *3* eBioscience, *4* Serotec, *5* Beckman Coulter, *6* Abcam, *7* R&D Systems

T cell cytotoxicity assay. For such assays T cell lines are required which are specific for a single antigen and that can be cultured for a longer period of time and to greater numbers. After the first round of antigen exposure and outgrowth of T cells, the lines need to be restimulated with antigen presenting cells (APC) in the presence of peptide to facilitate proper stimulation of the T cells. In this protocol autologous B cell lines immortalized with EBV are used as APC. Depending on the immunization protocol T cell lines can be generated against several MOG peptides, for example against MOG34-56.

Immortalized marmoset B cell lines are generated by infecting PBMC with EBV supernatant of the B95-8 strain. The B95-8 strain is derived from humans infected with EBV, who suffered from infectious mononucleosis [9]. This has been kept for several decades in cotton-top tamarins cells, also a New World monkey, and thereafter in marmoset cells. Infection with EBV immortalizes B cells, so they can be kept in culture for extended periods of time without the need for any other stimulation. These B cells can then be used as antigen presenting cells in various immunological assays, such as cytotoxicity assays, or to restimulate peptide-specific T cell lines.

To determine the cytotoxicity of peptide-specific T cell lines acquired from marmosets with EAE, a classic cytotoxicity assay is used, where immortalized B cells labeled with radioactive 51-chromium and MOG peptide serve as target cells for increasing amounts of T cells. Each ratio of T cells is tested in quadruplicate to correct for variation in the measurements. Next to each incubation with peptide-labeled B cell sample with T cells, also B cells incubated without T cells have to be tested to determine the spontaneous release of 51-chromium and in the presence of 2 % Triton X-100 to determine the maximum release of 51-chromium. These measurements are then used to determine the percentage of B cells killed by the antigen-specific T cell lines.

2 Materials

Prepare all mixtures in a sterile laminar flow hood at room temperature, and use only sterile equipment and disposables. All the steps involving physical handling of the marmosets, e.g., sedation and immunization, should be executed by personnel trained according to local animal ethic guidelines. All personnel working with radioactive material should have proper training and employ the necessary safety procedures when working with ^{3}[H]-thymidine or 51-Chromium. Contact the radiation safety officer for more information if unsure how to proceed.

2.1 EAE Induction Components

1. 10 ml glass vials.
2. Small stir bar (±1 cm).

3. Magnetic stirrer.
4. Buffered phosphate saline (PBS).
5. MOG34-56 peptide.
6. rhMOG (purified from *E. coli*).
7. IFA or CFA.
8. 1 ml syringe.
9. 26 G × ½″ needle.

2.2 Isolation of Mononuclear Cells from Peripheral Blood and Lymphoid Tissue Components

1. Ethylenediaminetetraacetic (EDTA) or heparin coated blood collection tubes.
2. Culture media: RPMI supplemented with 10 % fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μM Glutamax, 50 μM MEM Non-Essential amino acid solution, and 20 μM β-mercaptoethanol.
3. Cryotubes 2 ml.
4. Lymphoprep (AXIS-SHIELD, Oslo, Norway).
5. Leucosep tubes 12 ml (Greiner bio-one, Frickenhausen, Germany).
6. 5 ml syringe.
7. 70 μm cell strainer.

2.3 T Cell Proliferation by Thymidine Incorporation Components

1. Mononuclear cells (*see* Section 3.2).
2. Culture media: RPMI supplemented with 10 % fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μM Glutamax, 50 μM MEM Non-Essential amino acid solution, and 20 μM β-mercaptoethanol.
3. MOG peptide.
4. Concanavalin A (ConA).
5. Ovalbumin (OVA) or any other irrelevant protein or peptide.
6. 96-well plates U-bottom.
7. ^{3}[H]-thymidine, 50× diluted in culture media.
8. UniFilter-96 GF/C plate (Perkin Elmer, Waltman, USA).
9. Cell harvester for 96-well plates.
10. MicroScint-E (Perkin Elmer, Waltman, USA).
11. TopSeal-A 96 classic, seal for 96-well plate (Perkin Elmer, Waltman, USA).
12. Beta-counter.

2.4 MNC Phenotyping by Flow Cytometer Components

1. 96-well plates V-bottom.
2. 1 %BSA/PBS.
3. Monoclonal antibodies (*see* Table 1).

4. 1 % paraformaldehyde or BD Cytofix Fixation buffer.
5. Flow cytometer.

2.5 Antibody Measurement by ELISA Components

1. 96-well ELISA plates F-shape.
2. Bovine serum albumin (BSA).
3. Tween-20.
4. 3 M NaOH.
5. Antigen, for example MOG peptides.
6. Plasma samples.
7. Secondary antibodies: goat-anti-monkey IgM—alkaline phosphatase (AP) (Rockland Immunochemicals, PA, USA), rabbit-anti-human IgG—AP (Abcam, Cambridge, UK).
8. Substrate SIGMA Fast™ p-Nitrophenyl phosphate tablets.
9. Wash buffer: PBS containing 0.05 % Tween-20.
10. Blocking buffer: PBS containing 2 % BSA.
11. Dilution buffer: PBS containing 1 % BSA.
12. ELISA plate reader.

2.6 Generation of Peptide-Specific T Cell Lines Components

1. Mononuclear cells isolated from the spleen or lymph nodes (*see* Section 3.2).
2. Autologous immortalized B cell lines (*see* Section 3.7 and **Note 1**).
3. MOG peptides.
4. 24-well cell culture plate.
5. Interleukin 2.
6. Gamma or X-ray radiation cell irradiator.
7. Culture medium: RPMI supplemented with 10 % fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μM Glutamax, 50 μM MEM Non-Essential amino acid solution, and 20 μM β-mercaptoethanol.
8. IL2 starter medium: culture medium supplemented with 40 U IL2/ml.
9. T cell medium: culture medium supplemented with 20 U IL2/ml.

2.7 Generating B Cell Lines Components

1. Blood derived lymphocytes.
2. EBV supernatant strain B95-8.
3. Culture media: RPMI supplemented with 10 % fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μM Glutamax.
4. Phytohemagglutinin (PHA-M).
5. 24-well cell culture plate.

2.8 Assaying Cytotoxic T Cells Components

1. MOG peptide-specific T cell lines (*see* Section 3.6).
2. Autologous or allogeneic EBV infected B cells lines (*see* Section 3.7).
3. MOG peptides.
4. Culture medium: RPMI supplemented with 10 % fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μM Glutamax, 50 μM MEM Non-Essential amino acid solution, and 20 μM β-mercaptoethanol.
5. Chromium-51.
6. 96-well U-bottom plates with lid (Perkin Elmer, Waltham, USA).
7. Triton-X100, 2 % solution in culture medium.
8. Gamma radiation counter.

3 Methods

3.1 EAE Induction

1. Thaw rhMOG or MOG peptide and once thawed, keep it on ice.
2. Dilute the MOG antigen to a final concentration of 1 mg/ml in PBS.
3. Each animal has to be immunized with 100 μg MOG antigen in a final volume of 400 μl. The immunization emulsion consists of a 1:1 mixture of antigen in PBS and adjuvant (CFA or IFA) (*see* **Note 2**).
4. Pipet 200 μl CFA or IFA in a 10 ml glass vial followed by 100 μl PBS and 100 μl of MOG antigen from the stock solution of 1 mg/ml.
5. Gently stir this mixture for at least 1 h at 4 °C (cold room). The emulsion should turn an opaque white color (*see* **Note 3**).
6. After sedation, shave and disinfect the back of the marmoset.
7. Draw up the emulsion in the syringe directly after removing it from the stirrer. Place the vial with the mixture back on the magnetic stirrer, and keep it stirring during the entire immunization procedure. Place the needle on the syringe and remove any air bubbles.
8. Inject the mixture intradermally. Divide the emulsion over four spots of 100 μl each on the dorsal skin, two spots at the upper side and two at the lower side of the back.

3.2 Isolation of Mononuclear Cells from Peripheral Blood and Lymphoid Tissue

1. Collect blood into EDTA or heparin coated tubes from the femoral vein. Secondary lymphoid organs collected during autopsy should be stored in sterile culture media till processing (*see* **Notes 4–6**).

2. If needed, collect blood plasma before the isolation of MNC; spin blood for 10 min at 931 × *g*. Pipet off as much of the upper layer as required, and store it at −20 °C. This can be used to analyze compound, immunoglobulin, or cytokine levels.
3. Fill Leucosep tubes with 3 ml Lymphoprep, and spin down for 2 min at 1,126 × *g* (*see* **Note 7**).
4. After plasma removal, resuspend the remaining amount of blood with twice the original blood volume in culture media.
5. Pipet the diluted blood in Leucosep tubes containing Lymphoprep.
6. Spin Leucosep tubes with blood for 10 min at 1,126 × *g*.
7. With a plastic Pasteur pipet, transfer the lymphocyte layer from the Leucosep tube to a 15 ml tube containing 8 ml culture media.
8. Spin down for 10 min at 754 × *g* to wash away the Lymphoprep.
9. Resuspend pellet with ±10 ml fresh culture medium and spin down for 10 min at 754 × *g*. Repeat this step once to thoroughly wash the cells.
10. For the isolation of MNC from the spleen or lymph nodes, homogenize the organs with the plunger of a 5 ml syringe through a 70 μm cell strainer, creating a single-cell suspension.
11. Carefully wash the strainer with 5–10 ml culture media.
12. Layer spleen cells on the top of an equal volume of Lymphoprep, and spin down at 930 × *g* for 20 min without brakes.
13. Collect lymphocyte layer with a Pasteur pipet in a 50 ml tube containing 10 ml culture media and wash three times with ±30 ml culture media (*see* **steps 7** and **8**).
14. Wash lymph node suspension twice with culture media (*see* **steps 8** and **9**) (*see* **Note 8**).
15. Count live cells by using trypan blue or another method to discriminate between death and live cells.

3.3 T Cell Proliferation by Thymidine Incorporation

1. Resuspend isolated MNC in culture media and bring cells to a concentration of 1×10^6 cells/ml (*see* **Note 9**).
2. For each antigen you would like to test, pipet 200 μl/well of this cell suspension in triplicate in a 96-well plate. This results in 2×10^5 cells in each well.
3. Add 10 μl of 0.1 mg/ml MOG peptide, ConA, and OVA to the appropriate wells. This gives a final concentration of 5 μg/ml (*see* **Note 10**).
4. Incubate plate, wrapped in cling film, for 48 h at 37 °C.

5. If desired, collect 50 μl supernatant from each well, and store in a 96-well plate. Store these supernatants at −20 °C, also wrapped in cling film (*see* **Note 11**).
6. Add 0.2 μCi/ml of ^{3}[H]-thymidine to each well (*see* **Note 12**).
7. Incubate plate for 18 h at 37 °C.
8. To stop the proliferation of the MNC and to lyse the cells, place the plate for at least 3 h at −20 °C (*see* **Note 13**).
9. After defrosting the cell cultures, harvest the supernatant with the available cell harvester system. The procedure below is described for the UniFilter-96 GF/C plate.
10. Let the UniFilter-96 GF/C plate dry for at least 3 h at 37 °C.
11. Provide the bottom of the UniFilter-96 GF/C plate with the supplemented stickers.
12. Add 25 μl/well MicroScint-E to each well and seal the plate with TopSeak-A 96 classic film.
13. Measure the incorporated ^{3}[H]-thymidine with a beta-counter as a measure of proliferated cells.
14. Obtained count per minutes values can be expressed as stimulation index, the ratio of stimulated versus control wells (*see* **Note 14**).

3.4 MNC Phenotyping by Flow Cytometer

1. Plate 100,000–200,000 marmoset cells per well for each panel in a 96-well V-bottom plate (*see* **Note 15**).
2. Spin down the plate at 1,126 × *g* for 3 min.
3. Discard the supernatant by emptying the plate in the sink and softly tapping the plate on a piece of paper several times.
4. Wash cells once by resuspending the cell pellet with 150 μl 1 % BSA/PBS.
5. Spin down at 1,126 × *g* for 3 min and discard supernatant.
6. Resuspend cell pellet with diluted mAbs in 50 μl 1 % BSA/PBS (*see* **Notes 16–20**).
7. Incubate plate at 4 °C for 30 min.
8. Spin down plate at 1,126 × *g* for 3 min.
9. Wash cells twice with 150 μl 1 % BSA/PBS (*see* **steps 4** and **5**).
10. Resuspend the cell pellet in 180 μl 1 % BSA/PBS containing 1 % paraformaldehyde or 1× BD Cytofix Fixation buffer.
11. Fixate cells for at least 1 h at 4 °C.
12. Measure cells on the flow cytometer.
13. Analyze with the available software, for example FlowJo (TreeStar, OR, USA) or FacsDiva (BD Biosciences).

3.5 Antibody Measurement by ELISA

1. Coat the wells with the respective antigen, 5 μg/well in PBS in a total volume of 100 μl/well.
2. Incubate plate overnight at 4 °C (*see* **Note 21**).
3. Empty the plate softly on a piece of paper.
4. Block the wells with 200 μl/well with blocking buffer for 1 h at 37 °C.
5. Empty the plate (*see* **Step 3**).
6. Add plasma samples diluted in dilution buffer, 100 μl/well for 2 h at 37 °C (*see* **Note 22**).
7. Wash the plate five times with 200 μl/well wash buffer (*see* **Note 23**).
8. Add secondary antibody diluted in dilution buffer, 100 μl/well for 1 h at 37 °C.
9. Wash the plate five times with 200 μl/well wash buffer.
10. Dissolve a pNNP and a Tris Buffer tablet in 20 ml distilled water (substrate) (*see* **Note 24**).
11. Apply 100 μl/well substrate to each well and develop the plate at 37 °C in the dark till the positive control becomes yellow (*see* **Note 25**).
12. Stop reaction with 50 μl/well 3 M NaOH.
13. Read absorbance in a plate reader at 405 nm.

3.6 Generating Peptide-Specific T Cell Lines

1. Count the isolated mononuclear cells, and bring the cell suspension to a concentration of 10^6 cells/ml with culture medium. Pipet 1 ml of 10^6 cells/ml cell suspension in 8 wells of a 24-well plate (*see* **Note 26**).
2. Add 10 μg of MOG peptide to each well and wrap the 24-well plate in cling film.
3. Incubate the plate at 37 °C.
4. After 72 h, add 1 ml of IL2 starter medium to each well.
5. Place the cells back at 37 °C. From now on, assess the lines daily by microscopic examination.
6. Refresh the culture medium every other day.
7. As soon as the T cells start to grow out and cover approximately 75 % of the bottom, resuspend the wells and transfer 1 ml cell suspension to a new well.
8. Add 1 ml of T cell medium to both wells. Keep splitting the wells when necessary.
9. The T cell line can be kept in culture for approximately 2–3 weeks before restimulation is necessary.
10. When the T cells lines need to be restimulated, harvest all the cells from the 24-well plates in a 50 ml tube.

11. Count the number of living cells. Dilute the cell suspension to 0.4×10^6 cells/ml.
12. Harvest several million EBV transformed B cells and count (*see* **Note 27**). Irradiate the B cells with 50 Gray to prevent proliferation. Count the cells after irradiation, and dilute the cells to 0.4×10^6 cells/ml.
13. In a new 24-well plate, add 1 ml of T cell suspension (200,000 cells) and 1 ml of B cells suspension (200,000 cells) to 8 wells (*see* **Notes 26** and **28**).
14. Add 10 μg of MOG peptide to each well.
15. Incubate at 37 °C. From now on, proceed from **step 3**.
16. Antigen specificity of the line can be assessed by means of a T Cell Proliferation Assay (TCPA, *see* Section 4). Instead of adding 100,000 MNC to each well, add 40,000 T cells and 40,000 irradiated B cells.
17. As soon as a stable, peptide-specific T cell line is acquired it can be used in the desired immunological assay.

3.7 Generating B Cell Lines

1. Isolate lymphocytes from 2 ml EDTA or heparin blood and spin down in 15 ml tubes (*see* **Note 29**).
2. Resuspend pellet with ±2 ml EBV supernatant from the B95-8 strain (*see* **Notes 30** and **31**).
3. Incubate for 1.5 h at 37 °C (*see* **Note 32**).
4. Add equal volume culture media (±2 ml) containing 1 μg/ml PHA-M.
5. Plate cell suspension in a 24-well cell culture plate, 2 ml/well.
6. Incubate for 5 days at 37 °C.
7. Replace half of the media with fresh culture media.
8. Perform **step 7** twice a week till B cell lumps are formed (*see* **Note 33**).
9. If a well is 100 % confluent, divide cell suspension over 2 wells.
10. Repeat **step 9** till 8–16 full-grown wells are obtained.
11. Harvest all wells and transfer to a T25 cell culture flask.
12. Incubate at 37 °C and wait till the B cells are growing very well.
13. Replace in the meanwhile twice a week half of the media with fresh culture media (*see* **Note 34**).
14. If the B cells are increased in quantity and are growing well, it can be transferred to a T75 cell culture flask.
15. Scale up the number T75 cell culture flasks with B cells.
16. From now on B cells can be kept in culture forever.

3.8 Assaying Cytotoxic T Cells

1. Spin down 10^6 B cells in a 15 ml tube at 754 × *g* (*see* **Notes 35** and **36**).
2. Prepare a tube for each peptide against which cytolytic activity needs to be determined. Also include a tube with cells that will serve as unlabeled control cells.
3. Remove supernatant by pouring and resuspend the cells (*see* **Note 37**).
4. Add 10 μg peptide per 150,000 cells and proceed with the 51-chromium labeling.
5. Add 50 μCi of 51-Chromium (*see* **Note 38**) to each tube and place the tubes for 1 h at 37 °C.
6. Proceed in the meanwhile with the T cell lines.
7. The MOG peptide-specific T cell lines should be tested in different amounts to assess cytotoxicity. Count the T cell line and dilute the cells to 100,000 cells/ml (for a T cell:B cell ratio of 1:1), 400,000 cells/ml (T cell:B cell ratio 4:1), and 1.6×10^6 (T cell:B cell ratio 16:1) (*see* **Note 39**).
8. Add 100 μl of each T cell suspension in quadruplicate to the wells of a 96-well U-bottom plate.
9. For the spontaneous release, which serves as control wells, pipet 100 μl culture medium in 6 wells for each B cell sample.
10. Finally, pipet 100 μl 2 % Triton X-100 solution in other 6 wells for each B cell sample. These wells serve as maximum release of 51-chromium.
11. Proceed with washing the 51-chromium labeled B cells. Add 15 ml of culture medium to the tube and spin down at 754 × *g*. Discard the supernatant.
12. Repeat **step 5** twice and take up sample in 2 ml culture medium.
13. Count the living cells in each sample and dilute each sample to 50,000 cells/ml (*see* **Note 40**).
14. Add 50 μl (5,000 cells) of each peptide labeled B cell sample to each T cell ratio in quadruplicate, and to the spontaneous (no T cells) and maximum release (2 % Triton X-100) wells.
15. Spin down the plate for 15 s at 754 × *g*.
16. Incubate the plate for 5–6 h at 37 °C.
17. Harvest the supernatant which contains the 51-chromium, released by the B cells (*see* **Note 41**).
18. Read out on gamma radiation counter.
19. Determine the percentage of B cells killed for each ratio and peptide (*see* **Note 42**) by applying the following formula:

$$\text{Percentage cytotoxicity} = \frac{\left(\text{CPM}_{\text{sample}} - \text{CPM}_{\text{spontaneous}}\right)}{\left(\text{CPM}_{\text{maximum}} - \text{CPM}_{\text{spontaneous}}\right)} \times 100\ \%.$$

4 Notes

1. Autologous B cells are preferred; if these are not available allogeneic B cells could be used. Restimulation can also be performed with splenocytes or lymph node cells serving as APC.
2. Always make twice as much as necessary of the total immunizing mixture, because a lot of the preparation is lost during the process due to sticking to the glass vial.
3. Keep the mixture stirring while the marmosets are being sedated.
4. Due to ethical considerations, the maximum blood that can be collected in a month should not exceed 1 % of the body weight.
5. All animals have to be sedated during blood collection.
6. Secondary lymphoid organs should be collected after euthanasia by a well-trained dissector, as these can be hard to identify.
7. Use Lymphoprep for MNC isolation from blood and spleen cells; other methods such as Ficoll are suboptimal for the isolation of marmoset MNC.
8. Homogenized lymph node cells do not need to be layered on Lymphoprep for lymphocyte isolation.
9. Proliferation can be performed for all isolated mononuclear cells as described in Section 3.2.
10. OVA is used as negative control and ConA, a mitogen, as a positive control. Cultures without any antigen can also be taken along and considered as a negative control. It is advisable to leave at least one empty well between the wells cultured with ConA and the wells incubated with MOG peptide or OVA. Due to the high incorporation of thydimine in the ConA cultured wells, other wells can be contaminated. This will give a false-positive signal.
11. Supernatant can be collected for determination of cytokine profiles at a later time point. The supernatant of each triplicate can be pooled in one well in the new 96-well plate.
12. Make a working solution of diluted ^{3}[H]-thymidine and add 25 μl/well for a final concentration of 0.2 μCi/ml.
13. Plates can be kept for 1 or 2 weeks in the freezer due to the high stability of thymidine.
14. A stimulation index of 2.0 or higher is considered a positive response.
15. This method describes staining in a 96-well plate, but this can also be performed in small tubes, depending on the flow cytometer.

16. If necessary, perform a life/death stain before the first staining (after **step 3**), using for example LIVE/DEAD Fixable dead cell stain kit.
17. If more than one mAb is used for each well, make in advance a mixture of all mAb in a total volume of 50 μl 1 % BSA/PBS.
18. Perform a dose titration staining with each mAb to obtain the optimal concentration.
19. Prevent quenching of fluorescent dyes by protecting the plate from light with aluminum foil.
20. If mAb is not directly labeled with a fluorescent dye, perform after the first staining (from **step 9**) a second staining with an antibody that is fluorescent labeled and is directed against the species of the first antibody. For example, use a rabbit-anti-human if the antibody is directed against human.
21. Wrap plates in cling film during each incubation step to prevent dehydration of the wells.
22. Titrate plasma samples and secondary antibody for the right concentration, 1:200 and 1:2,000 is recommended, respectively.
23. Wash steps can be performed by using an ELISA washer or by a pipet.
24. Do not touch pNPP and Tris Buffer tablet without gloves.
25. Do not develop plate in an incubator.
26. Depending on the amount of cells available more or less wells can be started or restimulated.
27. In our experience six million cells (before irradiation) are sufficient to restimulate one T cell line.
28. If splenocytes or lymph node cells are used as an APC, use the following alterations to the protocol; irradiate the cells with 30 Gray, use 2×10^6 cells for restimulation of the T cells and 400,000 cells in a TCPA.
29. For isolation of lymphocytes from blood *see* Section 3.2.
30. EBV supernatant was collected at the Biomedical Primate Research Centre.
31. Perform all handlings in a laminar flow hood at ML-II level.
32. Do not tighten the cap of the 15 ml tubes at **step 3**.
33. It can take 2–6 weeks that B cell lumps are formed.
34. Freeze some vials with B cells as a back up after **step 13** and during **step 15**.
35. Autologous EBV infected B cells are preferred, but if these are not available the assay can be attempted with allogeneic B cells as target cells.

36. The amount of B cells can be adjusted depending on the conditions of the assays. If less ratios or peptides are used, less B cells can be used for labeling.
37. Take care to remove as much supernatant as possible, as this will benefit the 51-chromium labeling.
38. The current activity of the 51-chromium source can be determined by using the following formula: $N(t) = N_0(1/2)^{t/t1/2}$, where N_0 is the starting activity of the source, t the amount of days later, and $t_{1/2}$ the half life of 51 chromium in days, which is 27.8.
39. The T cell:B cell ratios can be adjusted to evaluate the cytotoxic capacities of the T cells in more detail.
40. The percentage of cell death can be expected to be as high as 50 %.
41. Depending on the type of gamma-radiation counter available the supernatant can be harvested in several ways. Check with the radiation safety officer which counter is available and how supernatants should be harvested.
42. The spontaneous release of 51-chromium by the B cells should not exceed 25 % of the maximum release. Higher percentages might mask any effect of the T cell lines and do not provide an accurate result.

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Part VI

Neuropathological Techniques

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Neuropathological Techniques to Investigate CNS Pathology in Experimental Autoimmune Encephalomyelitis (EAE)

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Abstract

Neuropathological techniques such as conventional and immunohistochemical staining of paraffin-embedded tissue sections are instrumental for identification and characterization of aberrations of organ architecture during human inflammatory disorders of the central nervous system (CNS) as in their animal models. Here we describe step-by-step protocols for tissue processing, sectioning, and conventional and immunohistochemical stainings to display as well as quantify CNS inflammation, demyelination, and neuronal damage in experimental autoimmune encephalomyelitis (EAE), an animal model of Multiple Sclerosis (MS).

Keywords: Experimental autoimmune encephalomyelitis, EAE, Multiple sclerosis, MS, Histology, Pathology, Neuropathology, Central nervous system, CNS, Myelin, Axon, Hematoxylin, Luxol fast blue, Bielschowsky, HE, LFB, PAS, Inflammation, Autoimmunity

1 Introduction

Neuropathological techniques have contributed significantly to our understanding of the CNS' architecture in diseased conditions. In the diseased CNS, conventional as well as immunohistochemical stainings can localize and characterize neurodegenerative and inflammatory processes. Histological hallmarks of neuroinflammatory disorders such as MS, which are the presence of inflammatory lesions, demyelination, and neuro-axonal damage (1–7) can be identified by neuropathological techniques such as hematoxylin/eosin (HE) staining (8, 9), luxol fast blue (LFB/PAS) staining for myelin (10), or Bielschowsky's silver staining for neurofilaments (11, 12). Routinely, we characterize inflammatory lesions and cellular alterations further by immunohistochemical stainings for T cells, macrophages, as well as axonal alterations (13, 14). EAE is an established animal model of MS. Immunization of susceptible animal strains with spinal cord homogenate, myelin protein, or myelin peptides emulsified most frequently in complete Freund's adjuvant generates an autoaggressive T helper cell response, which leads to a CNS inflammatory disease recapitulating certain histological hallmarks of MS lesions, depending on the model used

(15–19). EAE is frequently induced in mice and rats; therefore the protocols described here mostly focus on these two species. However, basic neuropathological techniques such as HE, LFB/PAS, and Bielschowsky's silver staining can be equally applied in experimental models of MS in other species, similar to human samples. Here we describe detailed step-by-step protocols for tissue processing followed by histological as well as key immunohistochemical stainings on paraffin sections to display and quantify CNS inflammation, demyelination, as well as neuro-axonal damage in the EAE model.

2 Materials

Make yourself familiar with the local regulations for waste disposal for all reagents used.

Prepare all solutions in ddH_2O or ultrapure water (18 mΩ cm at 25 °C) and analytical grade reagents.

2.1 Basic Equipment

1. Oven with adjustable temperature.
2. Surgical instruments: Fine forceps, blunt forceps, scissors, fine scissors, scalpels, or razor blades.
3. Histological glass/plastic ware: cuvettes, glass petri dishes, slide holders.
4. Organic solvents: ethanol, isopropanol, isopropyl alcohol, Xylol/UltraClear™ clearing reagent (Avantor, Center Valley, USA) (*see* **Note 1**).
5. Humified chamber (*see* **Note 2**).
6. Light-optical microscope equipped with a 10×, 20×, 40×, and 100× (Oil) ocular and with a color camera (e.g., Zeiss Axiophot). *Optional*: Digital slide scanner such as e.g. Mirax (Carl Zeiss AG, Jena, Germany) or Pannoramic (3DHISTECH, Budapest, Hungary).
7. *Optional*: Evaluation software such as ImageJ (NIH, Bethesda, MD, USA) or commercially available evaluation softwares such as Definiens Tissue Studio/Developer (Definiens, Munich, Germany) or similar.

2.2 Perfusion

1. 4 % Paraformaldehyde (PFA) solution: Heat 800 ml of phosphate buffered saline (PBS) in a glass beaker on a stir plate under a ventilated hood to approximately 60 °C. Attention: Do not boil. Add 40 g of PFA powder (Sigma-Aldrich, St. Louis, MO, USA). Incubate under continuous stirring for 3 h. Add carefully 1 N NaOH drop by drop until the solution clears. Cool the solution to RT and filter. Adjust the volume to 1 l by adding PBS. Check the pH, if necessary adjust to pH 7.0 using diluted HCl. Store at 4 °C for up to 2 weeks. For long-term storage, aliquot and freeze at −20 °C.

2. Peristaltic pump, *alternatively* luer lock syringes, with appropriate plastic tubes.
3. Collection receptacle with Styrofoam plate.
4. Animal anesthetic (e.g. Esconarkon ad us. vet.; Streuli Pharma AG).

2.3 Tissue Preparation and Processing

1. Embedding cassettes (e.g., VWR, Radnor, PA, USA).
2. Paraffination and Embedding system (e.g., tissue processing systems from Leica Biosystems, Wetzlar, Germany).
3. Stainless steel embedding molds (e.g., Sakura Finetek Europe, Leiden, Netherlands).
4. Heating plate.
5. Cooling plate.
6. Microtome (e.g., from Leica Biosystems, Wetzlar, Germany).
7. Water bath/glass petri dish filled with ddH_2O.
8. Superfrost Plus Glass Slides (Thermo Fisher Scientific, Waltham, MA, USA).
9. Glass coverslips (e.g., 20 × 50 mm, VWR, Radnor, PA, USA).
10. Xylol-based mounting medium (e.g., DePeX, Serva Electrophoretics GmbH, Heidelberg, Germany).

2.4 Histological Stainings

2.4.1 Hematoxylin/Eosin

1. Mayer's hemalum working solution: Dilute Mayer's hemalum solution (Merck KGaA, Darmstadt, Germany) 1:1 with ddH_2O and filter. Store the solution at RT. The working solution can be reused several times. Prepare a new solution when the staining becomes too faint.
2. 0.2 % HCl in EtOH: Dilute 2 ml concentrated HCl in 333 ml ddH_2O. Fill up to 1 l with EtOH and stir thoroughly.
3. 1 % Eosin: Dissolve 1 g Eosin G (Merck KGaA, Darmstadt, Germany) in 100 ml 70 % Isopropyl alcohol. Stir thoroughly and filter. Add a few drops of glacial acetic acid before use.

2.4.2 Luxol Fast Blue (LFB): Periodic Acid Schiff (PAS)—Staining

1. LFB solution: Dissolve 1 g luxol fast blue (e.g., Clin-Tech, Guildford, UK) in 1 l 96 % EtOH. Add 5 ml 10 % acetic acid, stir well, and filter the solution. The solution can be reused several times for up to 8 weeks. Prepare a new solution when the staining becomes too faint.
2. 0.05 % lithium carbonate: Dissolve 0.1 g Li_2CO_3 in 200 ml of ddH_2O. Always prepare fresh.
3. Schiff's reagent (Sigma-Aldrich, St. Louis, MO, USA).
4. 1 % HCl in 90 % isopropanol: Dilute 5 ml concentrated HCl in 100 ml ddH_2O. Fill up to 1 l with isopropanol and stir thoroughly.

2.4.3 Bielschowsky Silver Stain

1. 20 % silver nitrate solution: Dissolve 10 g $AgNO_3$ in 50 ml ddH_2O (always prepare fresh).
2. 32 % Ammonia (NH_3), concentrated solution.
3. Developer solution: Dilute 20 ml 37 % formalin in 100 ml ddH_2O. Add 0.5 g citric acid monohydrate and two drops concentrated nitric acid and stir well. Store developer solution at 4 °C protected from light for 4 to 6 weeks. Discard solution after usage.
4. 2 % sodium thiosulfate: Dissolve 2 g $Na_2S_2O_3$ ($\times 5$ H_2O) in 100 ml ddH_2O. Always prepare fresh.

2.5 Immunohistochemistry

1. 10 mM citrate pH 6: (a) Dissolve 21.01 g citric acid ($C_6H_8O_7 \times 2H_2O$) in 1 l ddH_2O. (b) Dissolve 29.41 g of trisodium citrate ($Na_3C_6H_5O_7 \times 2H_2O$) in 1 l ddH_2O. Add 9 ml of (a) and 41 ml of (b) to 450 ml ddH_2O. Verify pH.
2. Peroxidase blocking solution: 3 % H_2O_2 in PBS (dilute 1:10 from a 30 % H_2O_2 stock solution). *Alternative*: Dako® Peroxidase Blocking Solution (Dako, Glostrup, Denmark).
3. Fab-block: Fab Fragment Goat Anti-Mouse IgG (H + L) for staining of mouse tissue or Fab Fragment Goat Anti-Rat IgG (H + L) for staining of rat tissue (Jackson ImmunoResearch, West Grove, PA, USA).
4. Blocking solution: 10 % FCS (+0.1 % Triton X-100) in PBS (*see* Table 1).
5. Primary antibodies: Rat anti-human CD3e (clone CD3-12; cross-reaction with mouse CD3, AbD Serotec, Kidlington, UK), Rat anti-mouse CD107b (Mac3) (clone M3/84; Biolegend, San Diego, CA, USA), Mouse anti-rat CD3 (clone 1F4, AbD Serotec, Kidlington, UK), Mouse anti-rat CD68 (clone ED1, AbD Serotec, Kidlington, UK), Mouse anti-APP A4 a.a. 66-81 (clone 22C11, EMD Millipore, Billerica, MA, USA).
6. Secondary antibodies: Biotin anti-mouse IgG (GE Healthcare, Little Chalfont, UK), Biotin anti-rat IgG (DCS Diagnostics, Hamburg, Germany).
7. ExtrAvidin Peroxidase (Sigma-Aldrich, St. Louis, MO, USA).
8. DAB developer solution: Dissolve 2 g 3,3′-Diaminobenzidine (DAB) tetrahydrochloride hydrate (Sigma-Aldrich, St. Louis, MO, USA) in 80 ml PBS, and store 1 ml aliquots at −20 °C. For the developer solution, dilute 1 ml of DAB in 49 ml PBS and add 20 µl of 30 % H_2O_2. Prepare developer solution always fresh.

Table 1
Recommended antibodies and protocol details for staining of mouse and rat tissue

Staining	Primary antibody (dilution)	Clone	Antigen retrieval	Blocking solution	Secondary antibody (dilution)
T cells (mouse)	Rat anti-human CD3e (1:50)	CD3-12	Citrate pH = 6	10 % FCS + 0.1 % Triton X-100	Biotin anti-rat (1:500)
Activated macrophages (mouse)	Rat anti-mouse CD107b (Mac3) (1:100)	M3/84	Citrate pH = 6	10 % FCS	Biotin-anti-rat (1:500)
T cells (rat)	Mouse anti-rat CD3 (1:50)	1F4	Citrate pH = 6	10 % FCS	Biotin-anti-mouse (1:200)
Macrophages (rat)	Mouse anti-rat CD86 (1:500)	ED1	Citrate pH = 6	10 % FCS	Biotin-anti-mouse (1:200)
Axonal damage (mouse, rat, and human)	Mouse anti-APP A4 a.a. 66-81 (1:2,000)	22C11	Citrate pH = 6	10 % FCS	Biotin-anti-mouse (1:200)

9. *Optional*: Vector® M.O.M.™ Immunodetection Kit BASIC (Vector laboratories, Burlingame, CA, USA):
   (a) M.O.M.™ Mouse Ig Blocking Reagent: add 2 drops of stock solution to 2.5 ml of PBS.
   (b) M.O.M.™ Diluent: add 600 µl of protein concentrate stock solution to 7.5 ml of PBS.
   (c) M.O.M.™ Biotinylated Anti-Mouse IgG Reagent: add 10 µl of stock solution to 2.5 ml of M.O.M.™ Diluent prepared above.

3 Methods

All procedures should be carried out at room temperature (RT) unless specified otherwise.

3.1 Perfusion

All animal experiments and interventions have to be performed after permission and in accordance with local authorities. Therefore, recommended anesthesia protocols may differ depending on the local regulations.

Paraformaldehyde (PFA) is toxic. Work under a ventilated hood and avoid inhaling vapors during perfusion and preparation of tissues.

1. Prepare one bottle with 4 % PFA and one bottle with PBS (50 ml/mouse—200 ml/rat). Place a Styrofoam plate in a collection receptacle. Install a plastic tube attached to a syringe

with a needle or a butterfly syringe in the peristaltic pump and fill it with PBS. Important: Avoid air bubbles. Adjust pump velocity to approximately 2 ml/min and turn off. Place the tube into 4 % PFA.

2. Anesthetize the animal and verify anesthesia efficacy by squeezing the foot pads with tweezers.
3. Place animal on the Styrofoam plate and attach limbs to it with an adhesive tape. Open the animal's chest with small scissors. Insert the syringe needle into the left ventricle. Start the pump, and at the same time open the right atrium using small scissors. Perfuse the animal with approximately 30 ml/mouse or 150 ml/rat of 4 % PFA (*see* **Note 3**).
4. Prepare a piece of spleen and place it into a 15 ml falcon tube filled with 8 ml of 4 % PFA. Prepare the cord and the head (attached to each other), remove all fur, and place it into the same 15 ml falcon tube.
5. Postfix overnight at 4 °C. Then change to PBS for at least 3 h before tissue preparation.

3.2 Tissue Preparation and Processing

1. Carefully prepare brain and spinal cord from the perfused animals (*see* **Note 4**).
2. Following the scheme provided in Fig. 1, cut the cerebrum carefully in four slices, the cerebellum in two pieces and the spinal cord in eight to ten pieces of approximately 3–5 mm length (*see* **Note 5**).
3. Place all tissue pieces from one animal including spleen as internal staining control into an embedding cassette labeled with a block or identification number using a graphite pencil and close it. Place the embedding cassettes into a beaker containing PBS.
4. Perform dehydration and paraffination procedure according to the manufacturer's instruction of your paraffination and embedding system.
5. Preheat paraffin and embedding molds and the heating plate to approximately 70 °C. Place the embedding mold on the heating plate and fill it with liquid paraffin. Open the embedding cassette containing the paraffinized tissue pieces on the heating plate. Discard the lid, but keep the labeled cassette.
6. Following the scheme provided in Fig. 1 place cerebral and cerebellar slices, liver, and spleen as well as the spinal cord cone into the embedding mold. Take care about their orientation. Leave enough space for other spinal cord samples.
7. Put all other spinal cord pieces upright on the heating plate, in the same orientation as they have to be placed into the embedding mold. Carefully remove the embedding mold now containing all tissue pieces except the spinal cords from the heating plate. When the paraffin in the embedding molds starts to

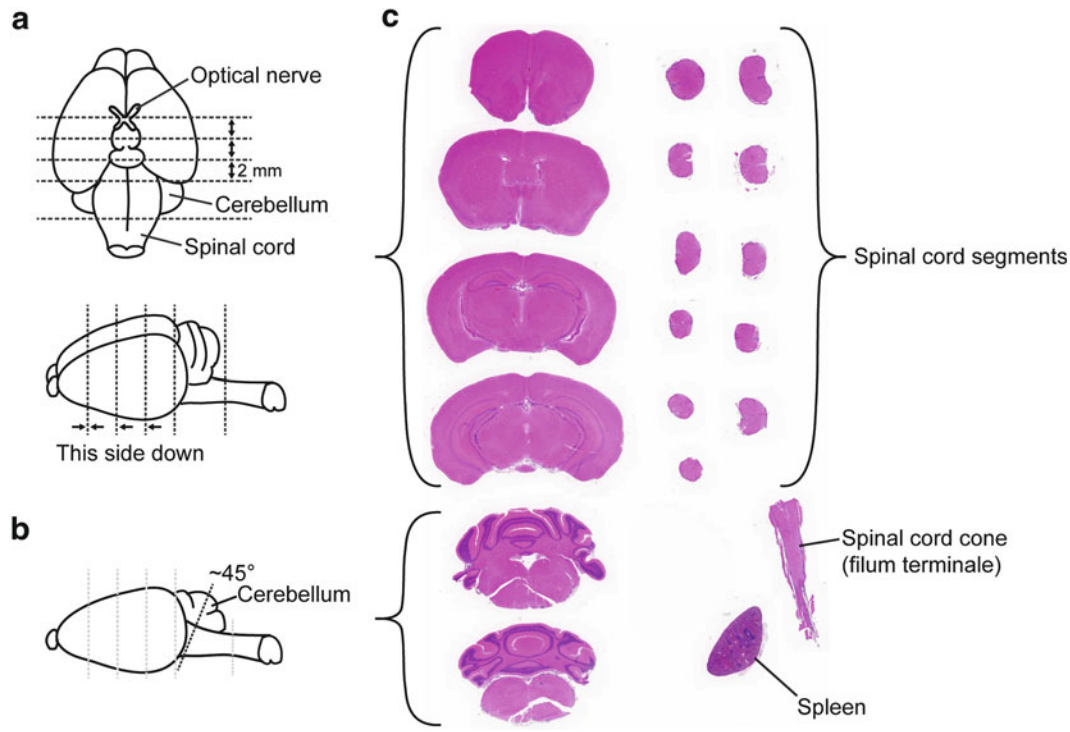

Fig. 1 Tissue processing and block arrangement. (**a**) The spinal cord is separated from the brain. By positioning the brain upside down, the crossing of the optical nerve is clearly visible. There you perform the first sagittal cut. Then, perform three additional posterior cuts 2 mm away from each other. (**b**) Cut the cerebellum into two pieces at an angle of approximately 45°. (**c**) HE stained paraffin section of an assembled tissue block

solidify from the bottom, place spinal cord pieces upright into it using preheated tweezers.

8. Place the labeled cassette on top, potentially add a bit more of the liquid paraffin on the top, and move the embedding mold to the cooling plate.
9. After approximately 20 min, paraffin blocks can be removed from the embedding molds using a knife. Verify orientation of brain and spinal cord samples (*see* Fig. 1 and **Note 5**).
10. Using a microtome, cut 2 μm sections and let them outstretch on water. Mount sections on Superfrost® Plus Glass Slides and let dry.

3.3 Deparaffination

1. Place slides with mounted paraffin sections (from here on referred to as slides) in a slide holder at 70 °C in the oven (20 min—overnight).
2. From now on work under a ventilated hood. Prepare glass receptacles containing the following solvents: 3× Xylol/Ultraclear, 2× 100 % EtOH, 95 % EtOH, 90 % EtOH, 70 % EtOH, 50 % EtOH and ddH_2O.

3. For deparaffination, place the slide holder with the preheated slides sequentially into the three glass receptacles containing Xylol/Ultraclear. Incubate for 10 min in each receptacle (*see* **Note 6**).
4. For rehydration, place slides sequentially into the glass receptacles containing decreasing concentrations of EtOH. At last place slide holder into the glass receptacle containing ddH_2O. Depending on the staining to be performed, rehydration might have to be stopped before complete rehydration (specified in protocols, *see* below).

3.4 Histological Stainings

3.4.1 Hematoxylin/Eosin (HE)

1. Deparaffinize and rehydrate sections until ddH_2O.
2. Filter Mayer's hemalum working solution. Incubate slides in Mayer's hemalum working solution for 30 s, and subsequently wash slides in ddH_2O.
3. Differentiate slides in 0.2 % HCl-alcohol (*see* **Note 7**).
4. Rinse slides in running tap water for approximately 10 min to "blue" staining. Verify nuclear staining under a light-optical microscope (*see* **Note 8**).
5. Incubate slides in 1 % Eosin for 5 min, and then rinse slides extensively in ddH_2O.
6. Rehydrate slides by incubating in increasing concentrations of EtOH (50 % EtOH, 70 % EtOH, 90 % EtOH, 95 % EtOH, 2× 100 % EtOH) followed by 2× Xylol/Ultraclear for 30 s each.
7. Using a xylol-based mounting medium coverslip slides, remove excess mounting medium by firmly pressing the coverslip on the slide.
8. Let dry at RT overnight or at 37 °C for 1 h.
9. Refer to Fig. 2 for anticipated staining results.

3.4.2 Luxol Fast Blue (LFB): Periodic Acid Schiff (PAS)

1. Deparaffinize and rehydrate sections until 90 % EtOH.
2. Filter LFB solution and add a couple of drops of glacial acetic acid.
3. Incubate sections in LFB solution in a cuvette at 60 °C between 1 and 2 days. Prevent evaporation of LFB solution by covering the cuvette with parafilm and lid.
4. Prepare a cuvette with 90 % EtOH and three big petri glass dishes (a–c) with ddH_2O (a), 0.05 % Li_2CO_3 (b), 70 % EtOH (c). Prepare an additional cuvette with ddH_2O to store slides after differentiation (*see* **Note 9**).
5. Transfer portions of four slides from the LFB solution to 90 % EtOH. Differentiate each slide individually by sequentially washing the slides in solutions (a)–(c): (a) Submerge slide with ddH_2O. (b) Wash in Li_2CO_3 until slide does not emit

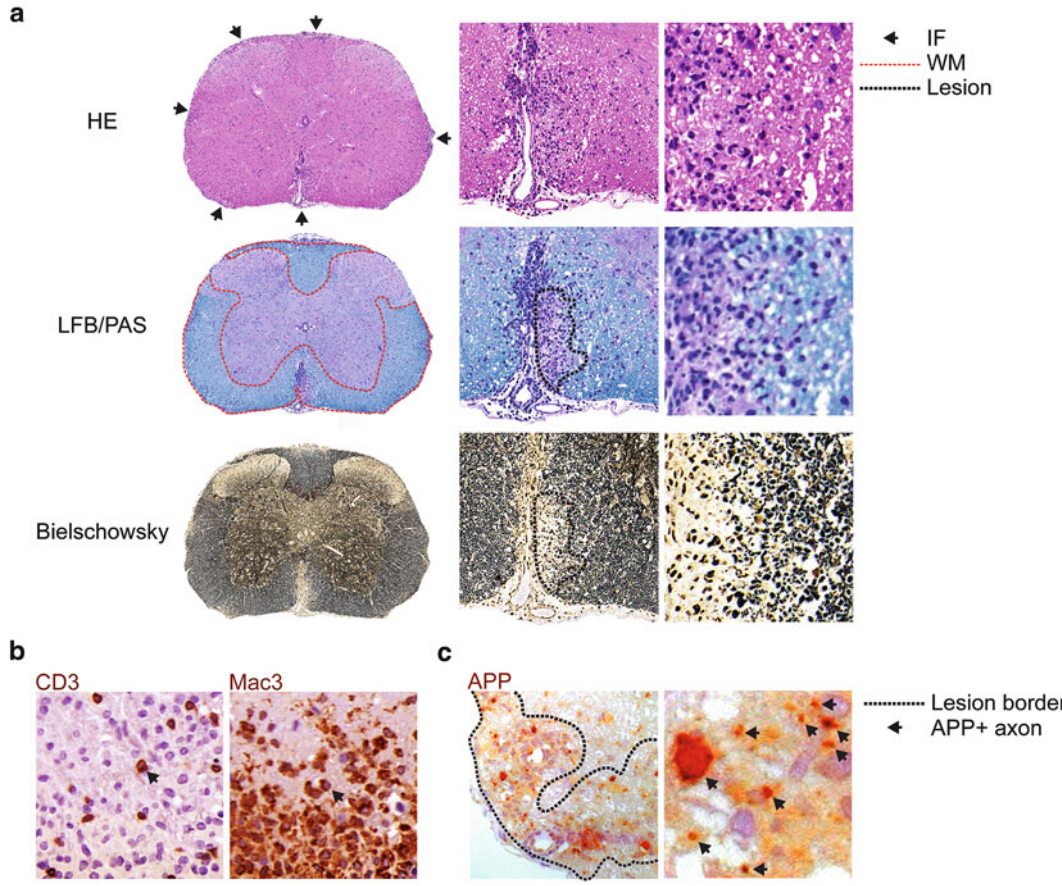

Fig. 2 Anticipated staining results. Representative staining of paraffin sections of mice during EAE (peak of disease). (**a**) *Left panel*: sagittal mouse spinal cord sections stained with HE (*top*), LFB/PAS (*middle*), and Bielschowsky's silver staining (*bottom*). Please note the presence of inflammatory foci in the HE staining and the discrimination of white and *grey* matter in the LFB/PAS staining. *Right panel*: Visualization of an inflammatory EAE lesion (*left*: overview, *right*: higher magnification 200×) by HE (*top*), LFB/PAS (*middle*), and Bielschowsky's silver stain (*bottom*). Please note the presence of compact small nucleated lymphocytes and macrophages visible in the HE staining, the demyelinated area visible in the LFB/PAS staining, and the profound axonal loss in the lesion visible in the Bielschowsky's silver staining. (**b**) *Left panel*: Anti-CD3 staining in an inflammatory spinal cord EAE lesion developed with DAB and counterstained with hemalum. T cells are identified as brown-colored nucleated cells (magnification: 200×). The arrow indicates a CD3+ T cell as example. *Right panel*: Anti-Mac3 staining in an inflammatory spinal cord EAE lesion developed with DAB and counterstained with hemalum. Activated macrophages are identified as brown-colored nucleated cells (magnification: 200×). Arrow indicates a Mac3+ activated microglia/macrophage as example. (**c**) Anti-APP staining in an inflammatory spinal cord EAE lesion developed with DAB and counterstained with hemalum. The punctuated Anti-APP staining identifies axonal APP accumulation due to impaired axonal transport as a surrogate for axonal damage (*left*: overview, *right*: magnification 630×). Please note that APP+ axons do not show hemalum counterstaining!

blue color anymore. (c) Wash slide in 70 % EtOH. Repeat steps (a)–(c) until the staining turns light blue. Perform microscopic control to verify myelin staining (e.g., strongly stained corpus callosum, only faintly stained cortex, and strong blue staining of spinal cord white matter, in contrast to adjacent gray matter) (*see* **Note 10**).

6. Transfer slides to a fresh cuvette with ddH_2O (prepared in 4).
7. Incubate slides for 5 min in 1 % periodic acid under a ventilated hood.
8. Rinse slides for 5 min in running tap water.
9. Rinse slides in three different cuvettes with ddH_2O to remove all tap water.
10. Prepare Schiff's reagent in a fresh dry cuvette (fume hood). Incubate slides for 20 min in Schiff's reagent. Verify under a light-optical microscope: faintly LFB-stained areas such as the cortex and spinal cord grey matter should have turned pink (*see* again **Note 9**).
11. Rinse slides with running tap water for 20 min. Transfer slides into ddH_2O.
12. Costain slides with Mayer's hemalum by dipping the slides five to ten times (depending on intensity of staining desired). Enhance nuclear staining by rinsing slides in running tap water for 5–15 min. Verify nuclear staining under a light-optical microscope.
13. Rehydrate slides in increasing concentrations of EtOH (50 % EtOH, 70 % EtOH, 90 % EtOH, 95 % EtOH, 2x 100 % EtOH), incubate slides for 30 s each in 2× Xylol/Ultraclear.
14. Using a xylol-based mounting medium coverslip slides, remove excess mounting medium by firmly pressing the coverslip on the slide.
15. Acquire images of spinal cord sections using a light microscope equipped with a camera or a slide scanner using at least at a ocular of 20×.
16. Analyze degree of demyelination as % demyelinated area/white matter (*see* below).
17. Refer to Fig. 2 for anticipated staining results.

3.4.3 Bielschowsky Silver Impregnation Stain

Work under a ventilated hood.

1. Deparaffinize and rehydrate sections. Store slides in ddH_2O until staining.
2. Prepare fresh 20 % $AgNO_3$ solution.
3. Prepare three cuvettes: (a) 20 % $AgNO_3$, (b) ddH_2O, (c) diluted ammonia (50 ml ddH2O + 1 ml NH_3 conc.).

4. Incubate sections in 20 % $AgNO_3$ (a) for 20 min. Sections turn light to dark brown depending on their thickness.
5. Rinse sections in ddH_2O.
6. Meanwhile add conc. NH_3 to 20 % $AgNO_3$ drop by drop (a) until the precipitate formed in step 4 has just disappeared (a*). Do not add excess ammonia, since it will inhibit impregnation.
7. Incubate sections in 20 % $AgNO_3$ + NH_3 (a*) for 15 min protected from light.
8. Rinse sections in 0.6 % NH_3 (b).
9. Add 1.4 ml developer solution to 50 ml 20 % $AgNO_3$ + NH_3 ((a*), used in step 7) (a**).
10. Transfer sections back to the cuvette (a**) now containing $AgNO_3$, NH_3, and the developer solution. Incubate for 1 min or less, since the reaction is very fast. Verify staining development under the microscope. Sections develop a caramel-brownish color in which axons/axon bundles turn black (*see* Fig. 2 and **Note 11**).
11. Stop development by rinsing slides in several changes of ddH_2O.
12. Incubate sections for 2 min in 2 % $Na_2S_2O_3$ to fix the staining.
13. Rinse sections in tap water.
14. Rehydrate slides in increasing concentrations of EtOH (50 % EtOH, 70 % EtOH, 90 % EtOH, 95 % EtOH, 2× 100 % EtOH), and incubate slides for 30 s each in 2× Xylol/Ultraclear.
15. Using a xylol-based mounting medium coverslip slides, remove excess mounting medium by firmly pressing the coverslip on the slide.
16. Acquire images of spinal cord sections using a light microscope equipped with a camera or a digital slide scanner using at least at a magnification of 40×.
17. Evaluate axon density in dorsal column and/or corticospinal tract as axons/mm^2 (*see* below).
18. Refer to Fig. 2 for anticipated staining results.

3.5 Immunohistochemistry

3.5.1 Basic Protocol

1. Deparaffinize and rehydrate sections until ddH_2O.
2. Perform antigen retrieval by heating slides in a microwave (800 W) in 10 mM citrate pH = 6 five times for 3 min each (*see* **Note 12**). Let the slides cool down in the same cuvette for 30 min before proceeding.
3. Wash slides in ddH_2O.
4. Buffer slides in PBS.

5. Block endogenous peroxidases: Place slides in a humified chamber and cover them with 3 % H_2O_2 in PBS or commercially available Peroxidase Blocking Solution. Incubate for 20 min.
6. Wash slides two times for 5 min each with PBS.
7. *Optional*: Place slides back into the humified chamber and cover them with 1:100 goat-anti-mouse IgG Fab fragments diluted in 10 % FCS (*see* **Note 13**). Incubate for 1 h.
8. Wash slides three times for 5 min each with PBS.
9. Place slides back into the humified chamber and cover them with appropriate blocking solution (*see* Table 1 and **Note 14**). Incubate for 30 min.
10. Remove blocking solution by tapping the slides on tissue. Do not wash.
11. Place slides back into the humified chamber and cover them with primary antibody diluted in 10 % FCS (*see* Table 1). Incubate overnight at 4 °C or 2–3 h at RT (*see* **Note 15**).
12. Wash slides three times for 5 min each with PBS.
13. Place slides back into the humified chamber and cover them with secondary antibody diluted in 10 % FCS (*see* Table 1). Incubate for 1 h at RT.
14. Wash slides three times for 5 min each with PBS.
15. Place slides back into the humified chamber and cover them with ExtrAvidin-Peroxidase diluted 1:1,000 in 10 % FCS. Incubate for 1 h at RT.
16. Wash slides three times for 5 min each with PBS.
17. Prepare fresh DAB developer solution as described in Section 2.5.
18. Place slides in a chamber and cover them with DAB developer solution. Control staining development under the microscope (*see* **Note 16**).
19. Stop and fix development by rinsing slides in ddH_2O.
20. Costain slides with Mayer's hemalum by dipping the slides five to ten times (depending on intensity of staining desired). Enhance nuclear staining by rinsing slides in running tap water for 5–15 min. Verify nuclear staining under a light-optical microscope.
21. Rehydrate slides in increasing concentrations of EtOH (50 % EtOH, 70 % EtOH, 90 % EtOH, 95 % EtOH, 2× 100 % EtOH), incubate slides for 30 s each in 2× Xylol/Ultraclear.
22. Using a xylol-based mounting medium coverslip slides, remove excess mounting medium by firmly pressing the coverslip on the slide.

23. Acquire images of spinal cord sections using a light microscope equipped with a camera or a digital slide scanner using at least at a magnification of 20×.
24. Analyze degree of demyelination as % demyelinated area/white matter (*see* below)
25. Refer to Fig. 2 for anticipated staining results.

*3.5.2 Adapted Protocol for Staining of Mouse Tissue with Primary Antibodies Derived from mouse: Anti-APP (Amyloid Beta (A4) Precursor Protein/ Alzheimer Precursor Protein) Using Vector® M.O.M.™ Immunodetection Kit (***see** *Note 17)*

1. Deparaffinize and rehydrate sections until ddH_2O.
2. Perform antigen retrieval by heating slides in a microwave (800 W) in 10 mM citrate pH = 6 five times for 3 min each (*see* **Note 12**). Let slides cool down in the same cuvette for 30 min before to proceeding.
3. Wash slides in ddH_2O.
4. Rebuffer slides in PBS.
5. Block endogenous peroxidases: Place slides in a humified chamber and cover them with 3 % H_2O_2 in PBS or commercially available Peroxidase Blocking Solution. Incubate for 20 min.
6. Wash slides two times for 5 min each with PBS.
7. Place slides back into the humified chamber and cover them with M.O.M.™ Mouse Ig Blocking Reagent (prepared as in Section 2.5). Incubate for 1 h.
8. Wash slides three times for 5 min each with PBS.
9. Place slides back into the humified chamber and cover them with M.O.M.™ Diluent (prepared as in Section 2.5). Incubate for 5 min.
10. Remove M.O.M.™ Diluent by tapping the slides on tissue. Do not wash.
11. Place slides back into the humified chamber and cover them with anti-APP diluted 1:2,000 in M.O.M. Diluent (*see* also Table 1). Incubate for 30 min at RT.
12. Wash slides three times for 5 min each with PBS.
13. Place slides back into the humified chamber and cover them with secondary antibody (M.O.M.™ Biotinylated Anti-Mouse IgG Reagent diluted 1:250 in M.O.M.™ Diluent (prepared in Section 2.5). Incubate for 1 h at RT.
14. Wash slides three times for 5 min each with PBS.
15. Place slides back into the humified chamber and cover them with ExtrAvidin-Peroxidase diluted 1:1,000 in 10 % FCS. Incubate for 1 h at RT.
16. Wash slides three times for 5 min each with PBS.
17. Prepare fresh DAB developer solution as described in Section 2.5.

18. Place slides in a chamber and cover them with DAB developer solution. Control staining development under the microscope (*see* **Note 16**).
19. Stop and fix development by rinsing slides in ddH_2O.
20. Costain slides with Mayer's hemalum by dipping the slides five to ten times (depending on intensity of staining desired). Enhance nuclear staining by rinsing slides in running tap water for 5–15 min. Verify nuclear staining under a light-optical microscope.
21. Rehydrate slides in increasing concentrations of EtOH (50 % EtOH, 70 % EtOH, 90 % EtOH, 95 % EtOH, 2× 100 % EtOH), incubate slides for 30 s each in 2× Xylol/Ultraclear.
22. Using a xylol-based mounting medium coverslip slides, remove excess mounting medium by firmly pressing the coverslip on the slide.
23. Acquire images of spinal cord sections using a light microscope equipped with a camera or a digital slide scanner using at least at a magnification of 400×.
24. Evaluate number of APP+ axons/mm^2 lesion (*see* below).
25. Refer to Fig. 2 for anticipated staining results.

3.6 Evaluation

During EAE, the extent of CNS inflammation varies during the disease course. At the peak of the disease (usually 3–5 days after the disease onset) inflammation is usually more pronounced than in chronic disease stages. Therefore, the time point when histopathological evaluation is envisaged has to be carefully chosen and adapted according to the scientific question addressed. In situations when, e.g., early inflammatory processes have to be evaluated, we recommend performing histopathological analysis at the peak of disease in which inflammation, demyelination, and quantification of T cells and activated macrophages can be easily performed. Similarly, the density of APP+ axons as surrogate for acute axonal damage is mostly pronounced at this early time point of the disease. Irreversible axonal loss visualized by Bielschowsky's silver staining can be detected during peak of the disease but also in later disease stages.

3.6.1 Inflammatory Index

1. For evaluation of an inflammatory index in *n* spinal cords, inflammatory foci (=areas of inflammation; IF) are counted for each spinal cord section individually using paraffin sections stained with HE (*see* Fig. 2 and **Note 18**). Albeit the "inflammatory index" represents a rather raw estimate of inflammation, it usually represents a good and fast method to evaluate the general inflammatory activity in the spinal cord.

2. The inflammatory index is calculated as follows (n = number of spinal cords analyzed):

$$\text{Inflammatory index} = \frac{\sum_{SC_1}^{SC_n} IF}{n}$$

3.6.2 Demyelination

1. Identify and measure the white matter (WM) area for each spinal cord section using paraffin sections stained with LFB/PAS (*see* **Note 19** and Fig. 2).
2. Identify and measure demyelinated areas for each single spinal cord section (*see* Fig. 2). Take care not to include meningeal infiltrates or perivascular edema. Especially the latter is pronounced in early disease stage and will thus result in an overestimate of demyelinated area when not excluded from the analysis.
3. Calculate the degree of demyelination for each spinal cord section and take the average over all section to determine the average degree of demyelination for one experimental animal and express in % if desired (n = number of spinal cords analyzed):

$$\text{Average degree of demyelination} = \frac{\sum_{SC_1}^{SC_n} \frac{\text{Demyelinated area}}{\text{WM area}}}{n}$$

3.6.3 T Cell (or Macrophage) Infiltration

1. Take overview pictures of whole spinal cord sections stained with anti-CD3 or anti-Mac3/CD68 and measure the area of white matter (WM area) of each single spinal cord section (which will allow you at the end to express cells per mm^2 white matter).
2. Identify T cells (or macrophages) as brown-colored nucleated cells in their respective immunohistochemical staining by manual counting (or automated detection if the required software is available) using a software platform (*see* Fig. 2 and **Note 20**). Count the number of T cells (or macrophages) in each spinal cord section individually.
3. Calculate the density of T cells (or macrophages) for each spinal cord section and take the average overall sections analyzed to determine the average infiltration density of T cells (or macrophages) as follows and express as cells/mm^2 (n = number of spinal cords analyzed):

$$\frac{\text{T cells (macrophages)}}{mm^2} = \frac{\sum_{SC_1}^{SC_n} \frac{\text{Number of T cells (macrophages)}}{\text{WM area}}}{n}$$

3.6.4 Axonal Loss (on Bielschowsky's Silver Stained Sections)

1. Identify and mark spinal cord lesions or defined areas and corresponding areas in control animals for axonal loss quantification (*see* **Note 21**).
2. Count axons in identified areas and calculate the axonal density for each area individually.

$$\text{Axonal density} = \frac{\text{Number of axons}}{\text{Analyzed area}}$$

3. Average all analyzed axonal densities and compare them to the axonal densities obtained from the same regions from control animals (*see* **Note 21**).

3.6.5 Acute Axonal Damage (on Anti-APP Stained Sections)

1. Identify demyelinated areas using an LFB/PAS stained section adjacent to the anti-APP stained section. Determine the area of each lesion.
2. Using high resolution images (min. 400×), alternatively directly under a light-optical microscope, quantify the number of APP+ axons in each lesion (*see* Fig. 2 and **Note 22**).
3. Calculate the average density of APP+ axons over all spinal cord sections analyzed as follows (n = number of lesions analyzed):

$$\frac{\text{Average number of APP}^{+}\text{axons}}{\text{mm}^2 \text{ lesion}} = \frac{\sum_{\text{Lesion}_1}^{\text{Lesion}_n} \frac{\text{APP}^{+}\text{axons}}{\text{lesion area}}}{n}$$

4 Notes

1. Xylol is environmentally toxic; therefore it is recommended to replace xylol with less toxic replacement solvents, such as Ultra-Clear™ clearing reagent (Avantor, Center Valley, USA) or equivalent.
2. The use of a humified chamber prevents evaporation of the applied solutions and drying of sections. Drying of sections will result in aberrant morphology and staining results. To provide additional protection from evaporation, slides can be carefully covered with parafilm once the antibody solutions have been applied.
3. A well-performed perfusion is essential for good tissue morphology, preservation of tissue antigens, and reproducible morphological analyzes. During perfusion, the peripheral blood should be removed from the animal almost completely, visible with the liver lightening up to a yellow color. A good perfusion will also lead to stiffening of the animal. If air bubbles emerge from the lung or the nose verify the location of the perfusion needle, since you might perfuse predominately via the

pulmonary circuit. Pay attention to a good perfusion speed: While a minimum of perfusion speed is necessary to thoroughly fix the tissues, a higher perfusion pressure will result in dilatation of vessels and aberrant tissue morphology.

4. Careful preparation of brain and spinal cord is critical. Use appropriate fine surgical instruments such as blunted forceps and small scissors. Take care not to damage the tissue. It is advisable to remove the outer meninges from the spinal cord, since they become very hard after paraffination and make it significantly more difficult to arrange the spinal cord segments in the embedding mold. When cutting the brain and spinal cord into segments, use a sharp razor blade or scalpel. Take care to not press the blade into the tissue, but to really cut it by moving the blade back and forth. A standardized protocol for tissue sectioning and block arrangement (*see* Fig. 1) leads to similar section planes in different blocks.
5. Paraffination preserves the tissue. If you encounter problems assembling and organizing the tissue segments in the embedding mold or during cutting, the tissue block can be melted and organized again without damaging the tissue.
6. A complete removal of all paraffin is critical for a successful staining. If small wax droplets are noticeable on the slide after deparaffination, dehydrate the tissue again in increasing concentrations of EtOH and incubate again in Xylol/UltraClear to completely remove the remaining paraffin. After that, rehydrate as described above and proceed.
7. The term "differentiation" refers to a specific washing step removing excess staining from background tissue, thereby "differentiating" the specific staining from the remaining tissue.
8. Hemalum solutions stain preferentially the nuclei of cells. The desired intensity of the staining depends on the tissue and personal preference. Thicker tissue sections will stain more intensely than thinner ones. Therefore, staining and differentiation steps have to be slightly adjusted by every lab. In any case, please note that after incubation in hemalum, differentiation and "blueing" in tap water, nuclei should be clearly visible and the tissue background should be clear. If the staining is too faint, slides can be washed in ddH_2O and stained again to increase staining intensity. The "blueing" process can be accelerated by using warm tap water.
9. For a successful LFB/PAS staining, the quality of the solutions is indispensable. Prepare Li_2CO_3 solution always fresh and change it after every ten slides. Verify the quality of the Schiff's reagent: The solution should be clear. A drop of Schiff's reagent on a white paper tissue should turn pink on its border.

10. The LFB/PAS staining should always be verified microscopically after differentiation with Li_2CO_3. While for example corpus callosum and the white matter of the spinal cord should be stained blue, the cortex and grey matter of the spinal cord should be clear (or show only faint staining). If the blue myelin stain is too faint, restain slides in LFB solution.
11. During Bielschowsky's silver staining, silver ions bind to axonal structures and are then reduced to visible metallic silver. The chemical reaction for the development of this staining is very fast and an accurate incubation time is very important. It is therefore recommended to start with one tissue slide to determine the exact reaction time resulting in black axons and axon bundles clearly distinguishable from a caramel-brown background. Note: While rinsing in ddH_2O stops the reaction in a reversible manner (slides can be developed further), incubation with $Na_2S_2SO_3$ fixes the staining.
12. Paraformaldehyde cross-links proteins during perfusion as well as post-fixation and can thereby sequester antigenic epitopes. Antigens can be retrieved by means of protein denaturation such as heat-mediated antigen retrieval using acidic citrate buffer. The exact temperature and heating time is critical for successful retrieval of antigens; therefore verify and adjust the power of your microwave. Accordingly, if lower power is used, the heating time might need to be prolonged. During boiling, take necessary precautions to prevent extensive evaporation of buffer from the cuvette to avoid drying out of the section. For example, use a lid (don't fix or screw!), refill cuvettes with ddH_2O after every 5 min of boiling and place the cuvettes into a beaker filled with ddH_2O at the bottom.
13. Application of goat-anti-mouse Fab fragment will block endogenous mouse immunoglobulins from being recognized by secondary antibody systems. Since the blood-brain barrier is damaged during inflammatory processes in the CNS, accumulation of blood-derived IgG can increase background signal in the CNS. This blocking step therefore reduces background staining especially in active inflammatory EAE lesions.
14. Serum immunoglobulins will occupy unspecific binding sites for immunoglobulins in the tissue and therefore limit background staining. For this blocking step it is recommended to use serum derived from the host of the secondary antibody used. The FCS used here closely resembles the goat immunoglobulins used as secondary antibodies, as alternative normal goat serum can also be used. By adding detergent, unspecific binding of antibodies can be further reduced; however, detergents can also inhibit the specific binding of antibodies to antigens. To increase blocking efficiency, blocking solutions

are not washed off, but simply tapped off. Further, antibody working solutions are prepared in 10 % serum to maintain blocking efficiency during incubation.

15. The incubation time of antibody dilutions depends on section thickness, degree of fixation, and localization of the antigen (time to penetrate the tissue). As a general rule, a longer incubation time at lower temperature (4 °C) will result in more specific and lower background staining than a shorter incubation time at RT.
16. The DAB developer solution should always be prepared on the same day and stored at 4 °C. Depending on the sensitivity of the secondary antibody systems and the developer solutions used, developing times can range from only seconds to several minutes. It is recommended to start with one slide to determine the exact reaction time for the best signal to background ratio. Development can be temporarily stopped by rinsing the slides in PBS. By applying new DAB developer solution, the slides can be developed further. When the development is satisfactory, slides should be rinsed in ddH_2O to denature HRP and fix the staining.
17. In our hands, the use of a mouse on mouse (M.O.M.) staining kit reduces the background originating from anti-mouse secondary antibodies recognizing mouse structures in mouse. Nevertheless, it is in principle also possible to follow the provided basic protocol and the protocol details provided for anti-APP staining in Table 1 for staining of mouse tissue, if appropriate caution is applied to staining development.
18. The quantification of inflammatory foci does not have to be performed on digital images; it can be quantified directly at a low magnification (100×) on a light-optical microscope. For the untrained eye, it might be difficult to detect small inflammatory foci as clusters of small compact nucleated lymphocytes in HE stained tissue. In that case, inflammatory foci can also be visualized by anti-Mac3 or anti-CD68 staining, which facilitate their detection.
19. For the quantification of demyelination, digital images are required. While it is easier to work with digital slides obtained from slide scanners or stitched images from a motorized microscope, quantification using microscope photographs in two different magnifications (100× for WM area and 200× for demyelinated areas) is also possible. Freeware such as ImageJ (for non-stitched images only) and Pannoramic Viewer (3DHISTECH, Budapest, Hungary) or commercial software can annotate areas and export in .csv or .xls formats.
20. The quantification of T cell and macrophage infiltrates can be in principle performed using the freeware mentioned above, but detection of stained cells has to occur manually, which is very

time consuming. There exist several software platforms which provide (semi-) automated cell detection and quantification which can significantly shorten the time of analysis, such as Definiens® Tissue Studio/Developer (Definiens, Munich, Germany) and others.

21. Please note that the axonal density in normal white matter in the spinal cord depends on the spinal cord segment (i.e., cervical, thoracic, lumbar spinal cord) as well as the region (e.g., dorsal column, corticospinal tract) analyzed. There are therefore two options: (a) Determine the axonal density inside lesions (identified by hemalum counterstaining or by HE or LFB/PAS staining on adjacent slides) and normalize to the axonal densities determined at the same spinal cord regions in control animals. (b) Determine the axonal loss only in specific spinal cord regions (e.g., dorsal column) on all spinal cord segments and normalize to the same regions in control animals. Take care to use images providing the necessary resolution to identify single axons in the region you choose, for some regions, 630–1000× magnified images are required. Please note that the resolution of single axons is also depending on the slide thickness. Preferentially thin paraffin slides of <2 μm thickness are to be used.
22. APP accumulation due to impaired axonal transport is used as a surrogate marker of axonal damage (20, 21). This accumulation often leads to an increased diameter of axons; thus the diameter of APP+ axons detected in EAE lesions can be enlarged up to several μm. Take care that APP+ structures quantified do not result from unspecific staining on lymphocytes (such as, e.g., plasma cells) or background staining (especially when staining mouse tissue). In all cases, hemalum counterstaining should be absent from APP+ axons (*see* Fig. 2).

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Neuropathological Techniques to Investigate Central Nervous System Sections in Multiple Sclerosis

Jan Bauer and Hans Lassmann

Abstract

Immunohistochemical techniques (IHC) and in situ hybridization (ISH) are widely used techniques to study the expression of proteins and messenger RNAs in tissues and are extremely important to confirm and interpret biochemical and molecular results from the same tissues. Investigation of human brain by IHC and ISH therefore still plays an important role in the elucidation of pathogenetic mechanisms in diseases such as multiple sclerosis. In this review we describe the processing of human brain tissues as well as basic and advanced immunohistochemical staining and ISH techniques used for neuropathological analysis of such pathological brains.

Keywords: Immunohistochemistry, In situ hybridization, Neuropathology, Multiple sclerosis, Paraffin embedded, Antigen retrieval

1 Introduction

During the last decades, major progress has been achieved regarding our understanding of the pathogenesis of inflammatory or degenerative diseases of the central nervous system through studies in experimental disease models. Although these studies without doubt have identified key disease mechanisms and paved the way for the development of new treatments, it also has become clear that experimental models only in part reflect the complex pathogenesis of chronic human brain diseases (1–3). Thus, it is necessary to validate the relevance of new pathogenetic concepts for human disease as far as possible through detailed and sophisticated analysis of metabolic or immunological changes in the respective patients and in particular within the brain lesions themselves. Molecular neuropathology and immunopathology are the disciplines, which can offer such an approach.

Such studies, however, are difficult in human brain diseases, since material for in-depth investigation is available only to a very limited degree. Material for this normally becomes available for research through the diagnostic procedure in brain biopsies and autopsies. On the other hand, the most interesting material is most

likely found in the archives of pathology departments where it is conventionally stored as formaldehyde-fixed paraffin-embedded blocks of tissue. This precludes a strategy, in which the process of tissue sampling, fixation, and storage is done according to the needs of the subsequent sophisticated methods of tissue analysis. Rather, methods of tissue analysis therefore have to be adapted and standardized in a way that allows maximal gain of information from archival material with suboptimal fixation and tissue preservation. During the last decades, major achievements have been accomplished to reach this goal (4–6). In this short review, we will describe the current status in handling and analyzing archival brain autopsy material using multiple sclerosis as a disease example.

2 Materials

2.1 Embedding Components

Paraffin: Histosec (Merck Darmstadt, Deutschland).

2.2 Antigen Retrieval Components

1. Heat-induced epitope retrieval buffers.
   (a) 0.1 M citrate buffer (citric acid in aqua dest) (AD), pH 6.0 (pH is increased with NaOH).
   (b) 0.05 M EDTA buffer in 0.01 M Tris buffer (pH is lowered to 9.0 with 1 M HCl).
2. Proteinase solution: 0.03 % proteinase (Type XXIV, Sigma) in 0.1 M PBS.
3. Formic acid: 100 % formic acid.

2.3 Immunohistochemistry Components

1. Antibody incubation buffer: All incubation steps are performed with DAKO washing buffer for automated systems (Washing buffer 10×, DAKO, Glostrup, Denmark) with addition of 10 % fetal calf serum (DAKO/FCS).
2. Production of biotinylated tyramide for catalyzed system enhancement.
   (a) Make a boric acid solution by adding 0.1545 g boric acid to 50 ml AD. Adjust to pH 8.0 with NaOH.
   (b) Take 6 ml of the boric acid solution and add 15 mg sulfo-NHS-LC-biotin.
   (c) Add 4.5 mg tyramine (Sigma).
   (d) Stir at RT over night.
   (e) Filter the solution (use 0.45 μm filter).
   (f) Aliquot (5–10 μl) and store at −20 °C.
3. Substrates.
   (a) 0.1 M Tris–HCl (pH 8.5) buffer: 12.1 g Tris in 1 l AD, set pH with HCl.

(b) 1 M Levamisole solution: 1 M Levamisole in Tris–HCl buffer.

(c) Fast Red substrate solution: Take 980 ml Tris–HCl buffer (0.1 M Tris, pH 8.0) and add 20 ml dimethylformamide, 1 ml from a 1 M Levamisole solution, and 200 mg Naphthol AS-MX phosphate. Store 100 ml aliquots at −20 °C. Before usage add 100 mg Fast Red TR salt. Filter the solution through a Whatman cellulose filter. Incubate sections at 7 °C for 10–45 min.

4. Fast Blue substrate solution.

(a) Solution A: Dissolve 12.5 mg Naphthol AS-MX phosphate in 615 µl dimethylformamide and add this to 100 ml of Tris–HCl buffer.

(b) Solution B: Dissolve 25 mg Fast Blue BB salt in 615 µl of HCl (2 N), add 615 µl sodium nitrite, and mix it gently.

(c) Add solution B to solution A and add 154 µl of the Levamisole solution. Filter through a Whatman cellulose filter. Incubate sections 10–45 min at 37 °C.

5. 0.05 % aminoethyl carbazole (AEC).

Stock solutions

(a) Solve 0.78 g AEC in 45 ml *N*,*N*-dimethylformamide.

(b) Make a 0.05 M sodium acetate buffer (6.8 g NaOAc in 1 l AD, adjust to pH 5.0 by addition of acetic acid).

Working solution: Add 1.15 ml stock AEC solution and 25 µl H_2O_2 (30 %) to 50 ml sodium acetate buffer.

2.4 In Situ Hybridization Components

1. DEPC (RNase-free) water.
1 ml diethylpyrocarbonate (DEPC) is added to 1 l bi-distilled water, incubated for at least 24 h at room temperature (RT). This is followed by autoclaving for 20 min at 120 °C to destroy the heat-sensitive DEPC. DEPC destroys RNases and is therefore toxic. In order to avoid autoclaving of each separate solution, all buffer solutions are prepared with this DEPC-treated water.

2. TBS buffer (pH 7.5).
For the stock solution, 60.57 g Tris base is solved in 500 ml DEPC water. The pH is adjusted to 7.5 with 400 ml of 1 M HCl. DEPC water then is added to a final volume of 1 l. Finally, 180 g NaCl is added. For the working solution, 50 ml stock solution is mixed with 950 ml DEPC water.

3. Tris buffer pH 8.0.
60.57 g Tris base is solved in 500 ml DEPC water. The pH is adjusted with 350 ml of 1 M HCl. DEPC water is added to a final volume of 1 l. For Tris 0.1 M working solution, the stock solution is diluted 1:100 with DEPC water.

4. 0.2 M Sörensen phosphate buffer (pH 7.4).
   13.8 g NaH_2PO4 and 71.2 g Na_2HPO4 are solved in 2.5 l DEPC water.
5. 4 % pH 7.4 paraformaldehyde (PFA) in phosphate buffer.
   40 g PFA (paraformaldehyde) is solved in 500 ml of 0.2 M Sörensen phosphate buffer. The solution is stirred at 70–95 °C until all PFA is solved. Addition of some drops of 1 N NaOH can be used to completely solve the PFA. Afterward fill up to 1 l with DEPC water and check the pH. This PFA solution is stored in the fridge at 4 °C.
6. Proteinase K solution.
   The concentration used for RNA retrieval depends on the fixation of the tissue. For optimally fixed experimental tissue or surgical biopsy material, a concentration of 20–50 μg/ml in TBS with 2 mM $CaCl_2$ is used. 10 mg proteinase K (from *Tritirachium album*) is solved in 1 ml TBS; 100 μl aliquots are stored at −20 °C. For 50 ml proteinase K working solution, one aliquot is thawed and added to 50 ml TBS with $CaCl_2$ at 37 °C. This mixture has a final proteinase K concentration of 20 μg/ml. For TBS with 2 mM $CaCl_2$, 0.294 g $CaCl_2$ (Merck) is solved in 50 ml TBS stock and 950 ml DEPC water.
7. 0.5 % acetic acid anhydride solution.
   1 ml acetic acid anhydride is added to 200 ml 0.1 M Tris.
8. Dextran sulfate solution.
   The viscous dextran sulfate solution increases the hybridization rate. 10 g dextran sulfate is solved in 20 ml DEPC water and stored in 1 ml aliquots at −20°C.
9. 2 % SDS solution.
   200 μg SDS (sodium dodecyl sulfate) is solved in 10 ml DEPC water and stored at RT.
10. 20× SSC solution.
    175.32 g NaCl and 88.2 g Tri-Na-Citrate (trisodium citrate dihydrate) are solved in 1 l DEPC water. For 1× SSC (1:50) and 2× SSC (1:100), 1× SSC is prepared by mixing 50 ml stock solution and 950 ml DEPC water, while 2× SSC is prepared by mixing 100 ml stock solution and 950 ml DEPC water.
11. 0.5 % sheared DNA.
    50 mg salmon sperm DNA is added to 10 ml water and stirred for 4 h. Then it is sheared by pressing through a 17-G hypodermic needle for four times. After this, the DNA is denatured by boiling for 10 min followed by rapid cooling in a mixture of ethanol and crushed ice. The cooking and annealing process is repeated once. Small aliquots (5–10 μl) are stored at −20 °C.

12. Deionized formamide.
    5 g deionizing resin (AG 501-X8 resin, Bio-Rad) is added to 100 ml formamide and stirred for 30 min. After this the solution is filtrated. 1 ml aliquots are stored at −20 °C.
13. Hybridization mix including probe.
    The hybridization mix including the probe consists of a fixed percentage of 10 % 20× SSC, 47 % deionized formamide, 10 % dextran sulfate, 2 % sheared 0.5 % DNA, and 1 % of the 2 % SDS. The remaining 20 % consists of the probe and DEPC water.
14. Roche blocking reagent buffer.
    12.114 g Tris and 8.766 g NaCl are solved in 1 l bi-distilled water.
15. Roche blocking reagent.
    5 g blocking reagent (Roche, 1096176) is solved in 1 l blocking reagent buffer and stirred at 50–70 °C. This solution stays cloudy. Aliquots of 50 ml in falcon tubes are stored at −20 °C.
16. NBT/BCIP buffer.
    12.114 g Tris, 5.844 g NaCl, and 10.165 g $MgCl_2.6H_20$ are solved in 1 l bi-distilled water; pH is adjusted to 9.5 with HCl 1 N.
17. NBT/BCIP working solution.
    700 µl dimethylformamide (DMF) is added to 300 µl bi-distilled water to become 70 % DMF. 100 mg 4-nitro blue tetrazolium chloride (NBT) is solved in 1 ml 70 % DMF. The NBT stock solution (protected from light) is stored at −20 °C. 50 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) is solved in 1 ml 100 % DMF and stored at −20 °C (protected from light). For the working solution, 225 µl NBT solution and 175 µl BCIP solution are added to 50 ml of the NBT/BCIP buffer.

3 Methods

3.1 Embedding of MS Tissue in Paraffin

Proper embedding of brain tissue in paraffin is an early and important part of neuropathological investigation. Too short processing times will lead to inadequate dehydration and tissue damage, while too long processing times will make the material brittle and difficult to cut. Depending on the size of the material, we use different embedding protocols. Routinely we embed small pieces of tissue (3.0 × 2.0 × 0.5 cm) or complete single or double hemispheric slices of approximately 1–1.5 cm thickness in paraffin with a low-melting point (Histosec, Merck). In the last few years, we however also have gained renewed interest in analysis of single or double hemispheric sections (Fig. 1a–c). Embedding of brain tissue is done

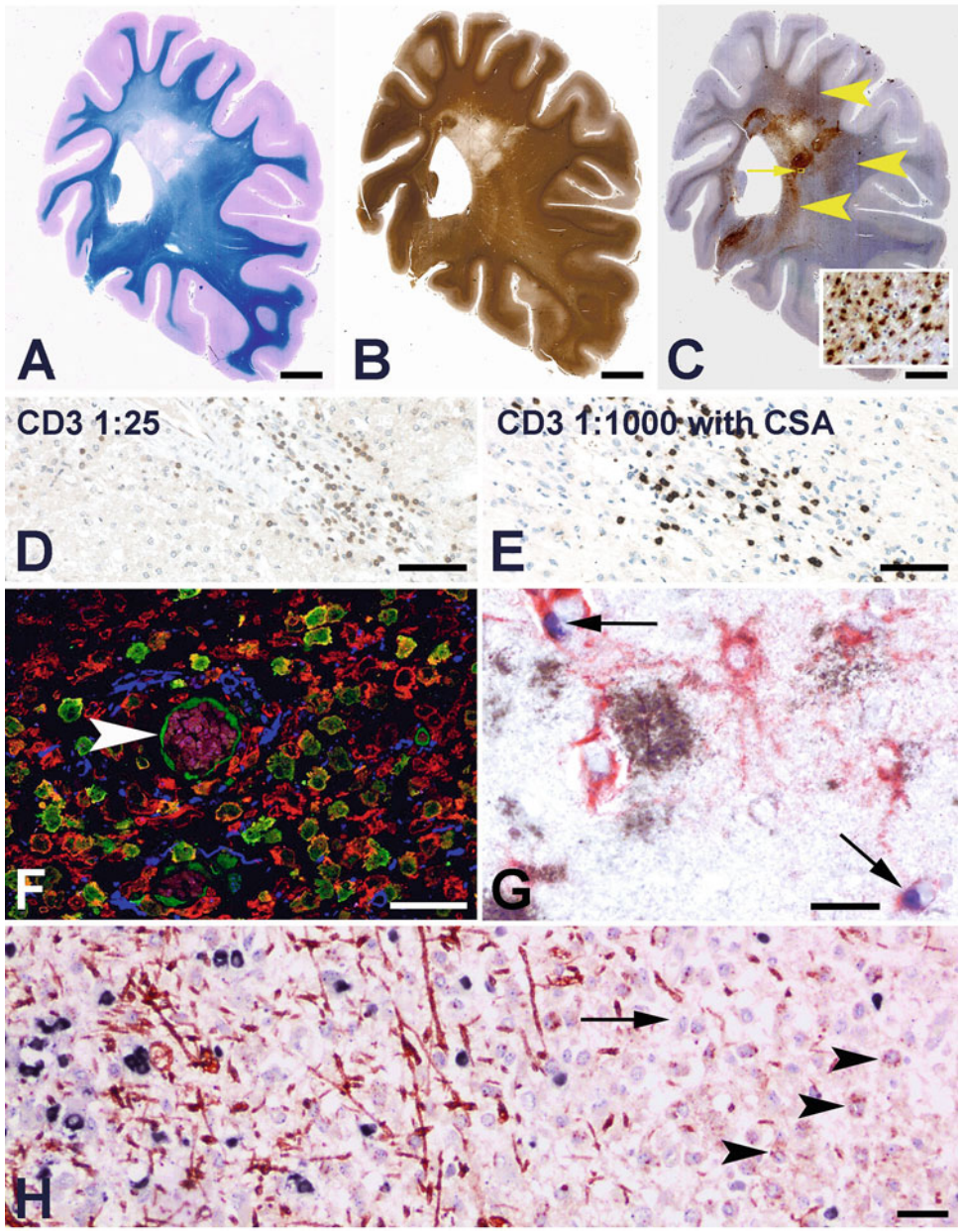

Fig. 1 Immunohistochemistry, confocal fluorescence microscopy, and in situ hybridization techniques. (**a**, **b**, **c**, bar: 1 cm) Hemispheric sections from the brain of an RRMS patient. (**a**) Luxol Fast Blue/periodic acid Schiff staining shows a demyelinating lesion in the white matter. (**b**) Staining for myelin oligodendrocyte glycoprotein (MOG) showing myelin loss in the same lesion. (**c**) Staining for CD68 shows strong upregulation in macrophages/microglia (*inset*) in the demyelinating area but also upregulation in the surrounding normal appearing white matter. (**d**, bar: 100 μm) Single immunohistochemical staining for CD3 (dilution 1:25) shows T lymphocytes in an acute MS lesion. Staining was performed with biotinylated anti-rabbit as secondary antibody, avidin peroxidase as third step, and DAB as substrate. (**e**, bar 100 μm) Same lesion as shown in (**d**). In this case CD3 was diluted 1:1,000, and the staining was enhanced with biotinylated tyramide. The CSA

in an automated system. Underneath are the dehydration and paraffin incubation steps which we find optimal for regular-sized tissue blocks (total 22 h) and double hemispheric blocks (total 78 h).

Station	Temp. (°C)	Solution	Normal-sized (2.0 × 3.0 × 0.5 cm) blocks	Double hemispheric blocks (1 cm thick) (h)
1	40	50 % ethanol	30 min	1
2	40	70 % ethanol	1 h	3
3	40	70 % ethanol	1 h 30 min	4
4	40	80 % ethanol	1 h 30 min	5
5	40	80 % ethanol	1 h 30 min	5
6	40	96 % ethanol	1 h	7
7	40	96 % ethanol	2 h	7

(continued)

Fig. 1 (continued) leads to immense signal enhancement with 40-fold reduction of the primary antibody. (**f**, bar 50 μm) Confocal fluorescence microscopy according to protocol in Section 3.3, step 3a. Staining of a demyelinating lesion in acute MS. Here, after HIER with EDTA pH 9.0, three primary antibodies derived from three species (mouse anti-transglutaminase 2, rabbit anti-Iba-1, and goat anti-GFAP) were incubated simultaneously. As a second step, we used biotinylated donkey anti-mouse, Cy3-conjugated donkey anti-rabbit, and Cy5-conjugated donkey anti-goat. The staining was finished with Cy2-conjugated streptavidin. The image shows Iba-1+ macrophages (*red*) and GFAP + astrocytes (*blue*). Transglutaminase 2 (*green*) is present in endothelial cells (*arrowhead*) and in various intensities in both Iba-1+ as well as Iba-1-macrophages but is completely absent in astrocytes. (**g**, bar: 20 μm) Triple staining on Alzheimer's disease brain, performed according to protocol in Section 3.3, step 3b. First, 1 h HIER was performed with a citrate buffer (pH 6.0). After this the primary antibody anti-Aβ antibody (Ab) (1:2,000 MAB1561, clone 4G8, Millipore, Billerica, MA, USA) was incubated ON. This was followed by biotinylated sheep anti-mouse (1:500 Jackson ImmunoResearch, West Grove PA, USA) and by avidin–peroxidase (Sigma, Germany). This staining was developed with DAB substrate. The DAB precipitate, due to steric hindrance, blocks binding of further antibodies to the anti-PCNA or the biotinylated secondary antibody. After washing, sections were incubated with anti-proliferating cell nuclear antibody (PCNA) Ab (1:25,000 DAKO M0879, Glostrup, Denmark) overnight, followed by donkey anti-mouse alkaline phosphatase-conjugated Ab, and NBT/BCIP substrate to produce a blue/purple stain (*arrows*). In order to inactivate the binding properties of the first round of antibodies and to retrieve additional GFAP epitopes, sections were then treated with EDTA (10 mM, pH 9.0) in Tris buffer for 30 min followed by incubation with rabbit polyclonal anti-GFAP (1:1,500, DAKO Z0334, Glostrup, Denmark). Subsequently, sections were incubated with alkaline phosphatase-conjugated donkey anti-rabbit (Jackson ImmunoResearch, USA) and developed with Fast Red substrate to stain astrocytes. (**h**, bar: 20 μm) ISH for proteolipid protein (PLP) in combination with IHC for the protein PLP. Shown is the edge of a lesion in a case of Balo's concentric sclerosis. At the *left side*, *black* PLP mRNA$^+$ oligodendrocytes are seen in between *red* PLP$^+$ IHC-stained myelin sheaths. At the *right side*, oligodendrocytes and myelin are lost. The *arrowheads* point at PLP$^+$ degradation products in macrophages in the demyelinated area. ISH was developed with NBT/BCIP, while the PLP ISH was developed with Fast Red. Hematoxylin was used as a nuclear counterstain

(continued)

Station	Temp. (°C)	Solution	Normal-sized (2.0 × 3.0 × 0.5 cm) blocks	Double hemispheric blocks (1 cm thick) (h)
8	40	96 % ethanol	2 h	7
9	40	Xylene	1 h	6
10	40	Xylene	1 h 30 min	6
11	61	Histosec (paraffin)	2 h	5
12	61	Histosec (paraffin)	2 h	6
13	61	Histosec (paraffin)	2 h	6
14	61	Histosec (paraffin)	2 h 30 min	10

3.2 Immunohistochemistry Preprocessing

For routine immunohistochemical stainings, paraffin sections are deparaffinized and dehydrated. In between these steps, we clear endogenous peroxidase. The protocol is as follows:

1. 2× 20 min deparaffinization in xylene.
2. 2× 96 % ethanol.
3. 20 min incubation in 0.2 % H_2O_2 in methanol.
4. 1× 96 % ethanol.
5. 1× ethanol 70 %.
6. 1× ethanol 50 %.
7. AD.

Antigen Retrieval

For most antibodies, antigen retrieval is obligatory to make antigens available for recognition. Most antibodies work after heat-induced epitope retrieval (HIER), while a smaller amount of antigens is retrieved after enzymatic digestion with enzymes such as proteinase, proteinase K, or trypsin. In addition, very few antibodies may benefit from antigen retrieval with formic acid.

1. HIER, rather than in a microwave, in our laboratory is performed in a household food steamer device (MultiGourmet FS 20, Braun, Kronberg/Taunus, Germany) by a 60 min incubation in a plastic coplin jar filled with the various retrieval buffers. Depending on the antibody, the used buffers are a citrate or EDTA–Tris buffer (pH 9. 0) as shown in Section 2. After steaming the slides are cooled down and transferred to TBS.

2. Proteinase treatment is performed by incubation of deparaffinized sections with proteinase (*see* Section 2) for 15 min at 37 °C.
3. Formic acid treatment is performed by incubation of sections for 15 min at RT.

3.3 Immunohistochemical Stainings

1. Immunohistochemical single stainings.
   By using antibodies against different cell types and structures, one can characterize the different lesions. The nuclear counterstain and weak background staining of the other structures in the tissue carry addition information such as the density of inflammation and the presence of edema. This information is mostly absent in fluorescence stainings which is why conventional (light microscopical) stainings are the first choice to start with. For stainings with single antibodies, we use a three-step method with peroxidase and DAB or AEC as chromogen or with alkaline phosphatase and Fast Blue or Fast Red as chromogen. After deparaffinization, blocking of endogenous peroxidase, and antigen retrieval, sections can be used for immunohistochemical stainings. Deparaffinized, untreated, or pretreated sections are transferred to TBS followed by the next protocol:
   (a) 10 min preincubation with DAKO/FCS.
   (b) Incubation with primary antibody in DAKO/FCS overnight (ON) at 4 °C.
   (c) Wash TBS 3×.
   (d) Incubation with biotin-conjugated secondary antibody (Jackson) for 1 h at RT.
   (e) Wash TBS 3×.
   (f) Incubation with avidin–peroxidase (Sigma, diluted 1:100) for 1 h at RT or avidin–alkaline phosphatase (Sigma, diluted 1:100).
   (g) Wash TBS 3×.
   (h) Development with DAB or alternatively Fast Red or Fast Blue substrate.
   (i) Wash with AD 3×.
2. Tyramide signal amplification (TSA).
   Many firms sell TSA kits which are used to enhance the detection of antigens. We use TSA with a variety of antibodies. In our hands, by using TSA, the dilution of the primary antibodies can be increased 5- to 20-fold with improved staining intensity (Fig. 1d, e). The TSA protocol and the production of biotinylated tyramide (7) originally are described by King et al. (6). Alternatively, tyramide can be coupled to a range of

fluorochromes as shown by Hopman et al. (8). The synthesis of biotinylated tyramide is a simple and cost-effective alternative to the purchase of TSA kits. For production of biotinylated tyramide, *see* Section 2.

Usage of Biotinylated Tyramide

(a) After step f of the IHC protocol (incubation with avidin peroxidase), wash with TBS.

(b) Dilute 5 µl of the biotinylated tyramide solution in 50 ml PBS and add 5 µl H_2O_2 (30 %); incubate sections for 10–20 min (*see* **Note 1**).

(c) Wash with TBS.

(d) Incubate sections with avidin–peroxidase (1:100) or alternatively with avidin–alkaline phosphatase (1:100) in DAKO/FCS for 30 min at RT.

(e) Wash with TBS.

(f) Develop with DAB, AEC, Fast Red, or Fast Blue substrate.

3. Immunohistochemical multiple labeling protocols.

(a) Multiple labeling with primary antibodies from different species.

Double-labeling studies mostly are performed with fluorescent dyes. However, double-labeling studies by light microscopy have certain advantages. First, it is easier to screen large areas and to trace specific areas. Second, it is easier to perform quantitative measurements. The most basic double labeling is performed with primary antibodies from different species and combination of a peroxidase and alkaline phosphatase detection system. These preferably are developed with aminoethyl carbazole (AEC) and Fast Blue as substrates. As a rule, the antibody, which in single stainings shows the weakest performance, is developed with a three-step biotin system and peroxidase as shown for single stainings. The "stronger" antibody is detected with an alkaline phosphatase-conjugated secondary antibody. In our protocol, as shown underneath, instead of peroxidase-conjugated avidin or alkaline phosphatase-conjugated secondary antibodies, also fluorescence-conjugated secondary antibodies or avidin-conjugated fluorescent dyes can be used (Fig. 1f). Since with fluorescence microscopy the number of labels are less restricted than for light microscopy (only peroxidase and alkaline phosphatase labeling, Fig. 1g), with fluorescence labeling detection of different antigens can be extended to three (Fig. 1f) or (theoretically) even more colors. Practically, however, the staining is restricted due to the limited availability of primary antibodies from various species and by the number of

fluorophores which can be used without overlapping wavelengths, in most cases excitation at around 405 nm (UV), 488 nm (green "FITC-like" dyes), 543 nm (red "rhodamine-like" dyes), and 633 nm (far red dyes). Underneath is the protocol which we use for both enzymatic as well as fluorescence labeling. Primary antibodies and secondary antibodies in this protocol are incubated pairwise as follows:

- 10 min preincubation with DAKO/FCS.
- Incubation with primary antibodies from different species (for instance, mouse and rabbit) in DAKO/FCS overnight at 4 °C.
- Wash TBS 3×.
- Incubation with biotin-conjugated secondary antibody (for instance, donkey anti-mouse) and alkaline phosphatase- or fluorescent dye-conjugated antibody (for instance, donkey anti-rabbit) for 1 h at RT (*see* **Note 2**).
- Wash TBS 3×.
- Incubation with avidin peroxidase (Sigma, diluted 1:100) or fluorescent dye-conjugated avidin (i.e., avidin Cy2, avidin DyLight 488, or avidin Alexa Fluor 488) for 1 h at RT.
- Wash TBS 3×.

In case of peroxidase and alkaline labels:

- Development of alkaline phosphatase with Fast Blue or NBT/BCIP.
- Wash with AD 3×.
- Development of peroxidase with AEC or DAB.
- Wash AD 3×.

(b) *Sequential staining with primary antibodies from the same species or antibodies which need different pretreatments.* Immunohistochemical stainings with antibodies from the same species are most precarious. There are a number of methods which can be used to stain two different antigens with antibodies raised in the same species. Firstly, in case of mouse primary antibodies, one can check whether the primary antibodies are in fact different immunoglobulin subsets (i.e., IgG1, IgG2a, or IgG2b) and therefore use subset-specific secondary antibodies. Secondly, stainings can be performed sequentially, meaning that a complete fluorescence or histochemical staining (primary antibody and secondary conjugated antibody [fluorescence or alkaline phosphatase]) is performed and repeated for the

second antigen by using a different fluorescent dye or a different enzymatic system (i.e., alkaline phosphatase label followed by peroxidase label). However, in most cases such a procedure will lead to false-positive double labeling due to cross-reactive binding of the secondary antibody from the second round of staining to the primary antibody of the first round of staining. This protocol therefore only can be used when the two antigens are in distinct different compartments (i.e., nucleus and cytoplasm or intracellular and surface) or in two different cell types (9). Instead of the above methods, we created a protocol in which the first antibody is developed with an alkaline phosphatase system by using Fast Red or Fast Blue as a substrate. Before we start with a second staining cycle, we then first treat the sections for at least 30 min with HIER with a citrate or EDTA buffer. As a result of this, primary and secondary antibodies from the first staining cycle are not detected anymore by secondary antibodies (10), while the Fast Red or Fast Blue deposits are unaffected and remain visible. In the second staining cycle, the primary antibody then is developed with a peroxidase system and AEC as a substrate. This system not only can be used as a double or triple staining with two primary antibodies from the same species but also can be used to combine two antibodies with different pretreatments (i.e., no pretreatment for the first primary and HIER for the second primary antibody, Fig. 1d). The protocol is as follows (washing steps with DAKO/FCS in between antibody steps are excluded):

- Deparaffinization including endogenous peroxidase quenching.
- No pretreament *or* HIER for 1 h, depending on the antibody.
- (First) Primary antibody overnight at 4 °C.
- Secondary alkaline phosphatase-conjugated antibody (1 h at RT).
- Development with FB.
- HIER with citrate or EDTA buffer for at least 30 min (*see* **Note 3**). Pretreatment can be up to 1 h when no pretreatment is used in the first staining.
- (Second) Primary antibody overnight at 4 °C (*see* **Note 4**).
- Biotinylated secondary antibody for 1 h at RT.
- Avidin–peroxidase for 1 h at RT.
- Develop with AEC or DAB.

3.4 In Situ Hybridization (ISH)

The in situ hybridization technique was originally developed by Pardue and Gall in 1969 (11). At that time, nucleic acids were labeled with radioisotopes, and autoradiography was the only technique for detection of the signal. In general, ISH in tissues can be used to confirm the presence of antigens/proteins at the mRNA level or can be used as an alternative, when detection of proteins with antibodies is not working. In MS research, detection of mRNA for myelin proteins such as proteolipid protein or myelin basic protein has been proven to be a valuable method to screen for oligodendrocytes in demyelinating lesions and reveal remyelinating oligodendrocytes in remyelinating lesions. In 1992 we published a nonradioactive ISH in which nucleic acids were labeled with digoxigenin and detected by immunohistochemistry with an anti-digoxigenin antibody and an alkaline phosphatase detection system (12). Since then we have used this technique on many occasions (7, 13–16). The technique itself has many advantages. No special laboratory equipment is required, there is no danger of radioactive pollution, probes are stable over a long period (up to some years), and there is a good signal–background ratio which implies optimal conditions for double staining. The staining signal is distinct and located in the cytoplasm (Fig. 1h). The exact protocol is given underneath. Note that the steps 1–17 are performed under RNase-free conditions:

1. Preparation of paraffin sections is performed with RNase-free (DEPC-treated) water.
2. Deparaffinization with xylene 3 × 20 min (first step can be done ON).
3. Hydration of sections: ethanol descending 96, 96, 96, 75, 50, 30%, H_2O.
4. Additional fixation of sections with cold 4 % paraformaldehyde in 0.1 M PBS for 20 min at RT temperature.
5. TBS 5× rinses.
6. Denaturize sections with HCl (0.2 M) for 10 min at RT.
7. TBS 3×–5× rinse.
8. Exposure of RNA by proteinase K treatment of sections for 20 min at 37 °C (*see* **Note 5**).
9. TBS 5× rinses.
10. TBS 4 °C for 5 min.
11. Acetylation with acetic acid anhydride treatment for 10 min during constant stirring (*see* **Note 6**).
12. TBS 3× rinses.
13. Dehydration with ascending ethanol 30, 50, 75, 96, 96, 96 % followed by chloroform.

14. Humidification of sections in a humid chamber at 55 °C for 30 min (*see* **Note 7**).
15. Incubation with the probe diluted in hybridization mix (about 15 µl/cm^2 tissue).
16. Cover with coverslip.
17. Linearization of RNA by incubation of sections on a heating plate at 95 °C for 4 min.
18. Humid chamber over at 65 °C ON.
19. Incubate sections in 2× SSC to detach coverslip.
20. High stringent washing steps.
21. 50 % formamide in 1× SSC for 3× 20 min at 55 °C.
22. One rinse with 1× SSC.
23. 2× 15 min 1× SSC at RT.
24. TBS 1×.
25. Incubation with Roche blocking reagent +10 % FCS for 15 min.
26. Incubation with alkaline phosphatase-conjugated anti-digoxigenin AP 1:500 in blocking reagent/FCS mix for 60 min.
27. TBS 5× rinses.
28. Incubation with NBT/BCIP at 4 °C in the dark, development up to 140 h.
29. AD rinse.
30. Nuclear counterstain with hematoxylin or start of immunohistochemical double staining procedure (*see* **Note 8**).

3.5 Discussion

Proper interpretation of histological slides for pathological analysis requires knowledge of tissue changes or the expression of candidate molecules in the context of entire lesions or even of the entire organ, such as the brain. Indeed, it proves to be advantageous to start the investigation with very large sections containing tissue of single (Fig. 1a–c) or, even better, double brain hemispheres (17, 18). In principle all immunohistochemical and in situ hybridization techniques, as described above, can be performed on such material, albeit at the expense of very high costs for antibodies and reagents.

The analysis of the general pathological context is extremely important for the correct interpretation of the expression of specific proteins or mRNAs. When the step of conventional immunohistochemistry is skipped and analysis is immediately started with fluorescence confocal laser microscopy, misinterpretations are commonly made. As an example, the absence of an immunofluorescent signal in a certain tissue region can be interpreted as the absence of expression within cells or other tissue components. Alternatively, however, it may be the result of a defect (hole) in

the section or due to global tissue loss in a destructive lesion. Therefore, proper interpretation requires a careful and detailed characterization of global pathological changes or the stage or activity within a given lesion (18). This can be done by analysis of alternate sections stained with conventional histological dyes for light microscopy. The use of conventional immunohistochemical techniques used for the detection of specific changes in protein expression combined with a conventional (counter) stain for cell nuclei however provides more information. This also applies for immunohistochemical double stainings using nonfluorescent conventional techniques. Such sections can easily be used for quantification and, for the question, if changes are just restricted to small focal areas or if they are a general feature of the pathological alterations (19). Quantification of cells or structures in neuropathology has increasingly become important to objectively analyze differences between normal and pathological brain. Quantification traditionally has been performed by using a morphometric grid (7, 20). In the last decennium, due to progress in computer technology and the development of specific quantification software such as the public domain program Image J (http://imagej.nih.gov/ij/), quantitative neuropathological analysis more and more is done by morphometric image analysis of scanned sections (7, 21). Conventional microscopical techniques here again have the advantage that quantification can be performed on images of large areas or even complete brain sections. For all these reasons, conventional techniques of immunohistochemistry should be the first choice of analysis, when starting with a new project or question.

Confocal laser microscopy, however, is a very useful tool for expanded secondary analysis. In general, confocal laser microscopy provides images with higher resolution, thus allowing in more detail the analysis of the subcellular localization of the respective antigen in question. A clear advantage of confocal laser microscopy is that it is very suitable and more reliable to study the expression of different antigens in the same cellular compartment (22), something which is much more difficult by conventional double staining. Furthermore, conventional techniques, in general, only allow to detect two to three different antigens simultaneously, while many more different antigens can be located by fluorescence confocal laser microscopy. This in particular is possible when directly labeled primary antibodies are available, when combinations with primary antibodies from different species can be made, or when primary antibodies from the same species can be neutralized by HIER treatment (7, 10, 23). Also in respect to quantitative measurements, confocal microscopy has some advantages. By selection of a specific pinhole within thicker slides, an optical section with a distinct thickness can be made and fluorescent signals quantified. This has the advantage that differences in section thickness between different sections can be excluded and small differences can be quantified reliably (22).

Finally, it has to be emphasized that a combination of techniques of in situ hybridization and immunohistochemistry is feasible (Fig. 1h) and highly useful for specific questions. This can be used to determine whether mRNA and protein expression concur or are temporarily separated. In addition, however, it can be used for a more general evaluation of tissue injury. As an example, in a variety of demyelinating conditions, the destruction of oligodendrocytes precedes the loss of myelin (24). This is, for instance, well shown in section stained by in situ hybridization of myelin protein mRNA and the respective protein by immunohistochemistry.

The sole availability of human formaldehyde-fixed and paraffin-embedded material has for long been a major hurdle for immunopathological and molecular pathological studies of human brain diseases. This unsatisfactory situation has radically been changed during the last years. As described above, we now have a large battery of antigen retrieval techniques, which dramatically increases the success of immunocytochemical studies on such material (25). In addition, this is boosted by the development of signal amplification techniques, which massively improves the sensitivity of the immunocytochemical staining (6, 25). Confocal fluorescence microscopy has benefited from the development of brighter, more stable, and much less bleaching fluorescent dyes which replaced, for decennia, the use of rhodamine and FITC (26, 27). Finally, antibodies from different species against the same targets are available from various companies. Many research projects can now be conducted on archival material, which was not possible even a few years ago. Nevertheless, the multitude of different technologies, all with their advantages and disadvantages, however, still require extensive technical standardization, before an actual research project can be started.

4 Notes

1. We incubate sections in a coplin jar filled with 50 ml of tyramide solution rather than incubate on the sections themselves. Since the solution does not include a detergent, it quickly retracts when incubated on the glass slides, leading to uneven or loss of signal at the edges. Before a new batch of biotinylated tyramide is used, this batch is tested in various concentrations (1:500, 1:1,000, 1:2,000) and various incubation times (10–20 min).
2. With this system one also can perform triple stainings: Instead of only one fluorescent dye-conjugated antibody, a secondary directly labeled fluorescence antibody against a third species can be used (for instance, primary antibodies from mouse, goat, and rabbit; secondary antibodies from biotinylated

donkey anti-mouse, Cy3-conjugated donkey anti-goat, and Cy5-conjugated anti-rabbit; and, as a third step, avidin-conjugated Cy2). We always use secondary antibodies raised in one specific single species (donkey) to avoid cross-binding of these secondary antibodies. For instance, simultaneous incubation with both goat anti-mouse and donkey anti-goat will lead to binding of the donkey antibody to the secondary goat antibody and thus to false double staining of the primary goat antibody.

3. Generally we add 30–45 min of the suitable HIER treatment (for instance, if in the first cycle 1 h HIER with citrate buffer is done and the second primary antibody works best with 1 h HIER EDTA 9.0, we add a 30 min HIER with EDTA 9.0).
4. At this stage it is also possible to incubate with two primary antibodies from two different species. In this case the staining can be continued with again one alkaline-conjugated and one biotin-conjugated secondary antibody. The staining then is finished by avidin–peroxidase and consecutive development with a different alkaline phosphatase substrate (Fast Red, when previously Fast Blue or NBT/BCIP was used) and DAB or AEC (Fig. 1g) (28).
5. Depending on the degree of formalin/paraformaldehyde fixation, proteinase K digestion can range from 10 to 100 μg/ml or, in case of tremendously overfixed material, up to 500 μg/ml. Too strong pretreatment however may lead to artifacts.
6. Acetic acid anhydride deactivates endogenous alkaline phosphatase; this step is performed during constant stirring with a magnetic stirrer in between 1-cm thick glass bars used as spacers at the bottom of a glass staining box (nr. H554.1, Carl Roth, Karlsruhe, Germany).
7. This step guarantees homogenous moistening of the slides.
8. After the ISH one can perform IHC, this however depends on whether the antibody tolerates the proteinase K digestion. This is the case for antigens such as proteolipid protein (PLP) or glial fibrillary acidic protein (GFAP). The staining is performed with an alkaline phosphate system with Fast Red as a substrate. As a result the ISH signal changes from blue/brown (NBT/BCIP substrate) into black which contrasts nicely with the red staining from the IHC (Fig. 1h).

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