Published in final edited form as:

Methods Mol Biol. 2013 ; 1013: 171–183. doi:10.1007/978-1-62703-426-5_11.

Visualizing Chemokine-Dependent T Cell Activation and Migration in Response to Central Nervous System Infection

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Abstract

In response to central nervous system (CNS) injury and infection, astrocytes, neurons, and CNS vasculature express several chemokines, including CCL21. Quantitative polymerase chain reaction (qPCR), western blot, and immunohistochemical methods can quantify mRNA and protein expression. However, these methods do not quantify chemokine bioavailability and bioactivity, variables modified by many environmental factors including composition of extracellular matrix (ECM). Here we illustrate how two-photon microscopy and carboxyfluorescein succinimidyl ester (CFSE or CFDA SE) labeling of T cells coupled with flow cytometry can be used as tools to assess chemokine-mediated regulation of T cell proliferation, activation, and migration.

Keywords

CFSE; Multiphoton microscopy; Cell migration; Cell proliferation; Second harmonic structures; Flow cytometry

1 Introduction

Chemokine expression is a critical aspect of the central nervous system (CNS) response to injury and infection [1–4]. Each chemokine serves as a recruiting, organizing, and activating agent for selected populations of immune cells expressing the appropriate receptors. However, distinguishing between immune responses for which a specific chemokine is necessary versus sufficient can be difficult due to the multiple inflammatory and cross-regulatory events triggered by injury and infection. An additional confound the complexity of the multiple ligand–receptor relationships between chemokines and their receptors. For example, CCR7 the high-affinity receptor for both CCL19 and CCL21, but each ligand triggers distinct biological effects [1–3]. Notably, while CCL19 is much more effective in supporting T cell activation and survival in vitro, CCR7-expressing T cells preferentially migrate toward CCL21 in a competing gradient in vitro chemotaxis assay.

In response to CNS *Toxoplasma gondii* (*T. gondii*), astrocytes express high levels of CCL21 while CCR7-expressing T cells infiltrate the CNS and home to foci of infection [3, 4]. Because CCL21 is a potent T cell chemoattractant, it is tempting to presume that CCL21 is sufficient to recruit T cells from the blood circulation and direct them to sites of infection, extrapolating correlative observations to causation. However, in support of this hypothesis,

CCL21 expression was found to be both necessary and sufficient to recruit CD4⁺ T cells into the pancreas in a murine model of autoimmune diabetes [5]. Therefore, to directly test the hypothesis that CCL21 performs the same role in the CNS, we generated transgenic mice in which astrocytes constitutively express slightly higher levels of CCL21 than observed in *T. gondii* infection [3].

Surprisingly, not only was there an absence of microglial activation, but also no T cells were found in the CCL21-expressing CNS. These data suggested that CNS-expressed CCL21 (a) was not detected by T cells located outside the CNS, (b) was not bio-active, or (c) was insufficient by itself to induce CCR7-expressing T cell infiltration into the CNS. Using CCL21 deficient and over-expression mouse models, we found that CCL21 while bioactive was insufficient to support T cell migration into the CNS. Conversely, we also found that CCL21 expression was required for CD4⁺ T cell migration from sites of extravasation to sites of *T. gondii* infection within the CNS [3]. In the following sections, we present two methods: (1) carboxyfluorescein succinimidyl ester (CFSE) labeling of T cells that allows simultaneous quantitation of cell proliferation and activation while brightly tagging cells for microscopic evaluation and (2) multiphoton-based microscopic imaging of cell migration within living tissue [5–10].

1.1 Using CFSE to Quantify Bioavailability of CNS-Expressed CCL21

Bioavailable CCL21 drives antigen-independent CD4⁺ T cell proliferation accompanied by upregulation of CD69 (facilitates T cell receptor responses), as well as adhesion molecules associated with extravasation and infiltration into the CNS [5]. For decades one of the most common methods for monitoring cell proliferation is based on quantifying radiolabeled nucleotide incorporation into the DNA of dividing cells [7–10]. However, this method is most appropriate in demonstrating proliferation at the level of an entire cell population. It does not provide information on the number of cell divisions completed per cell and the percentage of cells proliferating nor can it be readily combined with flow cytometric assays to quantify the expression of immune activation markers.

Alternatively, modified nucleotides such as bromodeoxyuridine (BrdU) can be fluorescently labeled so that proliferating cells can be identified in total cell populations by flow cytometric analysis or by fluorescent microscopy. This approach also allows simultaneous quantitation of markers of cell activation by flow cytometry, as well as provides spatial information regarding sites of proliferation when analyzing tissue sections. However, these approaches do not readily discern the number of cell division cycles by each fluorescent cell. In addition, detection in histologic section is difficult and may require signal amplification by a variety of methods. All of these limitations can be overcome by using CFSE [7–10].

CFSE is a nonfluorescent, cell-permeable fluorescein derivative. CFSE only becomes fluorescent after it diffuses into a cell's cytoplasm and its acetate groups are cleaved by cellular esterases [7–10]. The succinimidyl ester then binds free amine groups of other molecules within the cell resulting in long-lived fluorescent labeling of treated cells. These CFSE-modified molecules referred to as adducts are retained within the cell and distribute equally in daughter cells following cell division. Thus, with each cell division, CFSE fluorescence decreases by half. Enumeration of cell division cycles completed per cell is

readily analyzed by either flow cytometry of cell suspensions or confocal microscopy of living or fixed tissues using detectors and filters appropriate for fluorescein (FITC)-range fluorescent emissions. In our hands, 6–8 cell divisions are easily detected relative to fluorescence levels of unlabeled cell using standard flow cytometry (Fig. 1). In addition, this method is readily combined with other standard flow cytometry-dependent assays of cell activation/differentiation (using fluorescently conjugated antibodies or other dyes detected outside the FITC range of fluorescence emission). However, a few essential caveats need to be considered when designing experiments using CFSE-labeled cells.

First, although CFSE generates stable fluoresceinated adducts, the dye fades with time [7–10]. Therefore, to deduce fluorescence levels that corresponds to undivided cells, it is essential to fix (e.g., 1–2 % paraformaldehyde [PFA] in 1× phosphate-buffered saline [PBS]) and save an aliquot of the CFSE-labeled cells at 4 °C when initially labeling. Furthermore, autofluorescence varies as a function of cell type, activation state, or viability, or a combination thereof. Therefore, it is also essential to determine the autofluorescence of unlabeled cells at equivalent activation states in order to define the range that CFSE can be used as a marker of proliferation.

Second, the absolute fluorescence intensity level is directly proportional to the concentration of CFSE dye used to label a cell population [7–10]. During flow cytometry CFSE fluorescence is detected in the FL-1 channel. However, there is also substantial spread of fluorescein emission into the FL-2 channel that may potentially interfere with simultaneous detection of phycoerythrin (PE)-conjugated antibodies or other labels emitting in FL2 channel. This problem can be overcome in part by either flow cytometer hardware- or analytical software-based compensation. An alternative approach is to alter the initial labeling conditions to decrease the overall level of cellular fluorescence when initially labeling. In general, the magnitude of the desired CFSE fluorescence should be adjusted depending on the required experimental assay.

For example, cells are usually labeled at a final CFSE concentration of 0.5–2 μM for in vitro experiments. In contrast, cell tracking and generational analysis in cells adoptively transferred into a recipient animal may require labeling with 10–50 μM CFSE, with higher concentrations selected when substantial proliferation may occur. Lastly, it is important to note that high-labeling-concentration CFSE (>10 μM) can be toxic to some cells at rest or in specific activation states, such as highly activated T cells [11]. Therefore, viability analysis should be performed following initial CFSE labeling (e.g., Annexin V labeling of phosphatidyl serine on the outer surface of the plasma membrane) [11].

1.2 Using Live Cell Imaging in Brain Tissue

There are specific questions to be addressed when analyzing the immune response to infection as opposed to generating a pathological immune response—it is important to guide inflammatory cells to the site of infection as efficiently as possible to eliminate or at least contain the infection without damaging the surrounding tissue. There is much more known about the requirements to get cells into the brain and less about how they are directed once within it [11]. Chemokines and inflammatory molecules involved in this process are therefore of key interest.

Although several methods can be employed to study the infected brain's immune response, including immunohistochemistry and flow cytometry, the migration and behavior of cells within the brain can only be studied using multiphoton (MP) microscopy. There are many excellent reviews on the use of this type of microscopy [12, 13]. However there are three major aspects that make MP-imaging the only solution to investigating cell behavior within tissues: (1) It uses an infrared pulsing laser that has a longer wavelength allowing deeper tissue penetration; (2) a longer wavelength results in lower energy-lessening damage to the tissue, allowing longer imaging time; and (3) excitation only occurs at the focal plane as two photons are required to provide sufficient energy to excite the fluorophore. This prevents excitation above and below the focal plane allowing clear signal-to-noise ratio. These features of MP microscopy allow fluorescently labeled cells to be imaged at depths of up to ~300 μm for hours at a time.

The following method describes the procedure for tracking green fluorescent protein (GFP)-expressing T cells in an excised brain. Removal of the tissue is possibly the most straightforward procedure; however there are some caveats that can be overcome with choosing alternative procedures. Firstly, although the brain is kept alive in temperature- and oxygen-controlled environments, the tissue does not have a blood supply; therefore this procedure should not be used if the question being asked involves extravasation. Instead the vasculature can be kept intact by removing the skull and imaging the brain within an alive, terminally anesthetized mouse. Secondly, this is a terminal procedure! Therefore it is not possible to ask questions that may require imaging the same mouse over several days or weeks. Instead the skull can be left in place but thinned to allow a window for imaging and a specific area that can be returned to indefinitely. This method requires a lot more skill, technique, and equipment; however as it also leaves the surface of the brain intact, it avoids the activation of glial cells and, as a result, is essentially the gold standard for live cell imaging.

The advantages of removing the entire brain include being able to use second harmonic generation (SHG) to image structures in the brain. This is a phenomenon specific to MP microscopy and is the result of photons being combined on non-centrosymmetric structures and being scattered back at exactly half the wavelength and twice the energy. This has been observed with collagen structures in the lymph node, tumor environment, and granulomas [12, 13]. We have also observed inflammation-induced structures in the CNS that associate with migrating lymphocytes [6]. The intact skull would result in such a large SHG signal and prevent the collection of any signal from within the tissue.

Furthermore, although the lack of a blood supply hinders certain analyses, it reduces z-movement to a minimum. When imaging for long periods, slight changes in the z-position can result in the loss of the original imaging area and prohibit cell tracking. Lastly, the advantage of excising the brain allows the whole tissue to be imaged without confinement to a portion of the brain where the skull has been thinned. This is particularly useful when imaging infection as, unlike injury models, the infected area is not always known, so the ability to scan a relatively large area is beneficial.

The type of data obtained from these experiments will consist of the velocity and directionality of cells. Table 1 provides an overview of the types of analysis conducted and what the data may mean. For example, a T cell travelling at a speed less than 2 $\mu\text{m}/\text{min}$ is considered stationary and likely undergoes an antigen-dependent function, e.g., interaction with an infected cell. Also recent studies have demonstrated that T cells search the infected brain tissue in a traditional generalized Lévy walk dependent on chemokine signaling. This type of behavior maximizes the chances of the T cell finding a rare event—an infected cell. Thus, analysis of cell behavior is becoming more sophisticated but care should be taken to use a large data sample (number of cells and length of time) if generalized statements about whole cell population behavior are required.

Lastly, multiple cell labeling can be hugely advantageous to deciphering cell behavior. Examples include using mice with endogenously labeled microglia or astrocytes in the brain to allow cell–cell interactions to be measured or if the source of infection is also fluorescently labeled. However, the ability to image multiple fluorochromes will depend on the number of detectors available in the microscope setup and the ability to clearly distinguish overlapping emission signals. As this type of imaging becomes more widespread with the continued development of microscopes, it is likely that MP-imaging of multiple cells and their interactions will become possible.

2 Materials

2.1 CFSE Labeling and Detection

1. Purified cell population (*see* Note 1).
2. CFSE stock solution (10–50 mM) in dimethyl sulfoxide (DMSO) (*see* Note 2).
3. 1 $\times$ phosphate-buffered saline (PBS) or Hank's balanced salt solution (HBSS) plus 1 % bovine serum albumin (BSA) for labeling reaction.
4. Complete tissue culture medium, RPMI containing 10 % fetal bovine serum for washes and final resuspension (*see* Note 3).
5. Flow cytometer or confocal microscope equipped with lasers, detectors, and filters appropriate for fluorescein isothiocyanate (FITC) fluorescence.
6. Software to quantify fluorescence. CellQuest^{TM} and FlowJo can be used for acquired flow cytometric data (*see* Note 4) and Volocity (Perkin Elmer) can be used for acquired confocal/ multiphoton data.

¹CFSE labeling is most appropriate for cells that are fairly homogeneous in cell shape such as lymphocytes. As a consequence, the initial labeling of a homogeneous cell population will have a fairly uniform level of fluorescence per cell. When graphed, the entire population of labeled cells has a narrow range of fluorescence intensity (see narrow peak of labeled cells in Fig. 1). If cells are initially heterogeneous in morphology and size (such as observed with many macrophage-like cells or cultured cells), then the range of fluorescence intensity per cell will be fairly broad and thus interfere with definitive detection of proliferating cells with half of the fluorescence of undivided cells.

²Prepare the CFSE at a stock concentration 1,000-fold higher than the final usage concentration in DMSO. Aliquot CFSE solution into single-usage vials and store under desiccating conditions at -20°C . CFSE will hydrolyze quickly at room temperature with water present, and much more slowly at -20°C under desiccating conditions. Aliquoted stocks should be used for no more than 2 months.

³It is essential to maintain cells in single-cell suspension and completely mix the cells with the CFSE dye. The 1:1 ratio of cells to dye facilitates even labeling of cells such that a narrow range of fluorescence per cell is generated.

⁴Software programs such as ModFit can be used to estimate cell divisions in data derived from cells with an initial broad range of labeling intensities.

2.2 Live Cell Tracking in Brain Tissue

1. Mouse with appropriate fluorophore-labeled cells within the brain (*see* Notes 5 and 6).
2. Dissection equipment and complete tissue culture media. An MP microscope, i.e., one that has an infrared pulsed laser—this protocol uses a Zeiss LSM710 NLO microscope with Coherent laser with two non-descanning detectors (NDD) to detect SHG (425 nm) and GFP (530 nm) (*see* Note 7).
3. Live imaging setup attached to the microscope, i.e., temperature-controlled chamber and flow of oxygenated medium; *see* Note 8.
4. Analysis software, e.g., Volocity.

3 Methods

3.1 CFSE Labeling and Detection

1. Isolate T cells from lymph nodes, spleen, or specific tissues of interest. If activation, differentiation, or viability assays are required prior to CFSE labeling, cells can be treated with relevant antibodies or other labeling reagents at this point.
2. Suspend the cells at a concentration of 1×10^7 cells/ml or less in $1 \times$ PBS or HBSS containing 0.1 % BSA. Total reaction volumes should not exceed 4 ml in a conical 15 ml tube, so prepare cell suspensions at no greater than 2 ml each.
3. Prepare a solution of CFSE from DMSO stock containing 0.1 % BSA in $1 \times$ PBS at twice the final labeling concentration. For example, if you are labeling at 5 μ M, prepare a 10 μ M solution. Prepare the volume of CFSE equal to your cell volume above (no more than 2 ml per labeling reaction).
4. Add an equal volume of CFSE solution to your cell suspension. Mix gently and incubate for up to 10 min at 37 °C (*see* Note 4).
5. Immediately fill the labeling tube to the top with complete tissue culture medium and centrifuge for 7 min, $600 \times g$. Wash the cells three times with 3–5 ml of tissue culture medium at room temperature or 4 °C (*see* Note 9).
6. Resuspend at the concentration/volume appropriate for injection (50×10^6 cells/ml) or cell culture (2×10^6 cells/ml).

⁵Many strains of mice are available that have endogenously controlled fluorescence. A list available from The Jackson Laboratory can be found at the following Web site: http://jaxmice.jax.org/research/fluorescent_proteins_or_lacZ.html.

⁶Imaging and analysis are very time consuming (and expensive if paying for microscope time); therefore it is worth checking for fluorescent cells prior to imaging using another method (e.g., flow cytometry or immunohistochemistry) especially if the cells are being transferred or are not endogenous to the mouse. It is also useful to optimize the number of cells in the brain, e.g., too few migrating T cells will require several brains to generate significant data on cell behavior. Too many migrating T cells make analysis extremely difficult due to overlapping cell tracks.

⁷The fluorophore needs to be excitable by the two-photon laser and detectable by your microscope. Thus GFP with an excitation range of between 400 and 530 nm requires a two-photon laser wavelength of between 800 and 1,060 nm. At present, few two-photon lasers reach beyond 1,100 nm and the majority do not function above 950 nm.

⁸Anything other than excised tissue will require an upright microscope or an inverter. The temperature-controlled chamber is often set up so that a 60 mm Petri dish fitted with a 1.5 mm diameter cover glass floor fits in the center. The thickness of the cover glass is crucial.

⁹To reduce the amount of unbound CFSE in cells, incubate the cells at 37 °C for 5 min between the second and third washes; this facilitates the release of free non-reacted CFSE by diffusion out of the cells for removal in the final wash.

7. Immediately fix an aliquot of the labeled cells in 1–2 % paraformaldehyde solution (in 1× PBS) to provide a CFSE fluorescence standard in nondividing cells.
8. Perform flow cytometry on a small aliquot of labeled cells (50,000–100,000 cells) to determine the range of CFSE fluorescence intensity per cell and general cell viability with a flow cytometric instrument capable of detecting FL1/FITC-type fluorescence.
9. Cells are now ready for transfer into cultured tissues, living recipient mice, or for in vitro use in activation and proliferation assays. Following completion of the experiment, harvest the cells of interest from culture or the lymph nodes, spleen, or other tissues of the recipient animal. Analyze both the recovered cells and the stored fixed cell samples (*see step 7* above) obtained at the time of initial labeling at the same time (by flow cytometry or microscopy).

3.2 Live Imaging in Brain Tissue

1. Excise the mouse brain and place in a temperature-controlled chamber.
2. Set the objective (*see Note 10*).
3. Look through the eyepiece using normal argon lamp fluorescence and focus. Superficial brain autofluorescing blood vessels should be clearly seen, providing targets to facilitate focus.
4. Find your field of view—this will depend on your experimental question; however this include an area of fluorescent cells. At this point, it is often possible to detect highly migratory cells through the eyepiece alone.
5. Switch to the two-photon setup by turning down the argon lamp and switching to NDD (*see Note 11*).
6. Confirm signal by live tissue scanning (*see Note 12*).
7. Set up a z-series. The z-series should be thick enough to capture a significant portion of tissue allowing the SHG structure to be noted and so that migrating cells can be followed for a significant length of time. We suggest a z-series of six steps separated by 6 μm giving a total z-stack of 30 μm . Increasing the step size may result in a migrating cell being lost between slices; increasing the total height of the stack will increase the length of time it takes to image—crucial when combining with a time-series.

¹⁰If using an upright setup, a dipping objective is required with appropriate working distance; otherwise a 40× water objective is needed to provide the best field of view in our experience. When using water objectives, make sure that the water is added to the objective prior to mounting the sample as it can be challenging to add afterwards. Avoid using medium containing pH indicators as this can generate autofluorescence.

¹¹NDDs are highly sensitive allowing maximum sample signal detection. However, it also means they can pick up ambient light, including light from the computer monitor. Minimize sources of light in the room and cover detectors and microscope with blackout curtains.

¹²At this point the SHG signal can be seen as long fibers or waves especially in the area of blood vessels—presumed to be collagen. GFP-positive cells will be clearly observed alone in the single channel—small autofluorescing green blobs are often seen but show up in all channels and are much smaller than T cells (Fig. 2).

8. Set up a time-series. The time-series needs to be long enough so that the migration and behavior of cells can be analyzed. The time-series is based on how many consecutive repeats of the z-stack are to be imaged. Therefore the length of the movie generated will depend on the time it takes to image one z-stack. A minimum of 20 time points should be imaged to generate enough data to make conclusions about velocity and direction of migrating cells.
9. Run the time-series (*see* Note 13).
10. Analysis of cell migration requires specialized software. Few programs allow tracking of cells in four dimensions (x, y, z, and time). We have successfully used Volocity that allows manual and automatic cell tracking (*see* Note 14).
11. Using Volocity, import the new image sequence from acquisition files.
12. Modify auto contrast and color as desired (e.g., green for GFP).
13. Go to “measurements view” and “measure all time points.”
14. Find objects—this can be done in several ways depending on the level of background, number of cells, and measurements wanted. The following is an example:
15. Find by % intensity (cut off lower and upper 5 % and look at histogram).
16. Then exclude by size (most cells are $>200 \mu\text{m}^3$).
17. Track items—here essentially the formula for finding the next position of the cell defined—shortest path is normally the best using $<10 \mu\text{m}$ distance.
18. Once tracks are obtained, highlight the track objects, go back to the image view, and play the movie checking that the tracks make sense.
19. Save as a measurement item. Each tracked object will have a mass of data values presented as a table including average velocity, meandering index, and displacement. The resulting data can be copied and pasted into an external software spreadsheet for subsequent analyses.

Acknowledgments

Development of assays and generation of data supported by a University of California—Riverside Chancellor’s strategic initiative and National Institutes of Health grants NS071160A and NS072298A. The authors acknowledge Corinne Ploix for contributions toward standardization of flow cytometric protocols and Sean Wilson and Monica Rubalcava (Loma Linda University School of Medicine) for contributions toward optimization of multiphoton microscopy protocols.

¹³ When running the time-series, it may be apparent that there is z-movement. This may happen especially in thick tissues such as the brain as it settles or if using a live mouse setup due to respiratory movements, etc. Pick a landmark in a z-plane and watch for changes or appearance in a different plane. If this occurs, then the movie should be quickly stopped. Reset the z start and stop positions to account for the movement and restart the movie. During data analysis, files can be combined to join the break in the movie sequence.

¹⁴ When using the manual tracking feature, the cell’s center needs to be indicated at every time point using x-, y-, and z-planes. This introduces user error or bias but may be required sometimes when there are many overlapping tracks. When using the automated tracking settings, the parameters have to be defined carefully to find only objects that are cells. Following tracking, observe selected tracks to ensure that they adequately account for the expected cell behavior.

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Fig. 1.

CFSE labeling to quantify CCL21-dependent CD4⁺ T cell proliferation

Fig. 2.

Snapshot image showing SHG signal (*blue*), eGFP (*green*), and tracks of migrating T cells (*pink*)

Table 1

In vivo measurements using multiphoton microscopy

In vivo measurements	Definition	Interpretation
Velocity	Speed of cells	Cell velocity decreases in the presence of antigen; velocity <2 $\mu\text{m}/\text{min}$ is considered stationary
Distance	Total distance travelled from point A to B	Distance covered may include repetitive behavior and is not the best indicator of how directed a cell is
Displacement	Actual distance travelled from point A to B	Fast highly directed cells are expected to have greater displacement indexes
Meandering index	Measurement of directionality	Distance travelled/displacement: The closer the meandering index is to 1.0 the more directed the cell is travelling from point A to B
Clustering	Group of cells with distinct behavior	Clusters of slow or stationary cells implies antigen-specific responses
SHG signal	Volume of reticular fibers	Inflammation-induced reticular fiber migration of cells is associated with these fibers