Video Article

Quantifying Human Monocyte Chemotaxis *In Vitro* and Murine Lymphocyte Trafficking *In Vivo*

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Abstract

Chemotaxis is migration along a specific chemical gradient[1]. Chemokines are chemotactic cytokines that promote cellular trafficking with anatomic and temporal specificity[2]. Chemotaxis is a critical function of lymphocytes and other immune cells that can be quantitatively assessed *in vitro*. This manuscript describes methods that permit the evaluation of chemotaxis, both *in vitro* and *in vivo*, for diverse cell types including cell lines and native cells. The *in vitro*, plate-based format permits the comparison of several conditions simultaneously in real-time, and can be completed within 1-4 h. *In vitro* assay conditions can be manipulated to introduce agonists and antagonists, as well as differentiate chemotaxis from chemokinesis, which is random movement. For *in vivo* trafficking assessments, immune cells can be labeled with multiple fluorescent dyes and used for adoptive transfer. The differential labeling of cells allows for mixed cell populations to be introduced into the same animal, thereby decreasing variance and reducing the number of animals required for an adequately powered experiment. Migration into lymphoid tissue occurs in as little as 1 h, and multiple tissue compartments can be sampled. Flow cytometry following tissue harvest allows for a rapid and quantitative analysis of the migratory patterns of multiple cell types.

Video Link

The video component of this article can be found at https://www.jove.com/video/56218/

Introduction

Robust immunity requires the complex temporal and spatial coordination of a myriad of cell types in order to respond appropriately to injury, infection and generate self-tolerance. Several dozen chemokine receptors and their corresponding ligands have been discovered and characterized providing molecular mechanisms by which specific cells can be directed into a specific tissue at a specific time. Thus, studying chemotaxis and migration is an indispensable component of immunology research. Indeed, the described *in vitro* assay was recently used as a screening tool to identify a chemotactic cofactor that accelerates chemotaxis of T-cells toward C-C chemokines 19 and 21[3]. The purpose of the methods described here are to permit quantitative assessments of immune cell chemotaxis *in vitro* and *in vivo*.

The Boyden (cell migration and invasion) chamber assay is an inexpensive, reproducible, and rapid method for assessing cell migration[4,5]. In the standard assay, the upper chamber is seeded with cells, and is separated by a porous insert from a lower chamber, into which the cells migrate. At the desired time, cells that have migrated to the underside of the insert can be fixed and stained for quantitation by light microscopy. However, such measurements constrain data collection to a single end point, which precludes dynamic data collection and can require extensive optimization to determine the optimal time point for analysis. Here, several adaptations are described that permit real-time, quantitative and multiplexed measurements of chemotaxis *in vitro*.

For *in vivo* studies, a functional end point is used, namely the specific accumulation of cells in a given tissue compartment. Pre-labeled donor cells are introduced into recipient animals through adoptive transfer. These donor cells can subsequently be identified by flow cytometry after recipient tissue harvest. Also presented is a co-labeling strategy that allows for the determination of trafficking of different cell types within a single recipient animal. This method eliminates the inter-animal variation from cell injection, and accounts for physiologic inter-animal variability.

Protocol

All procedures were approved by Rockefeller University's Institutional Animal Care and Use Committee. All animals were housed under specific pathogen-free conditions.

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1. *In Vitro* Chemotaxis

1. Culture THP-1 cells[6] in Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with 10% fetal bovine serum (FBS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 25 mM) (complete media), maintaining the density between 0.5-1.0 x 10^6 cells/mL.
2. In a 35 mm cell culture dish, label 3.5 x 10^5 THP-1 cells per condition with calcein acetoxymethyl (calcein-AM; 2.5 µM) or other suitable fluorescent dye in complete media at 37 °C for 30 min.
   NOTE: Dyes must be cell membrane-permeable and compatible with live-cell labeling.
3. Pre-warm a plate reader to 37 °C.
   NOTE: In addition to temperature control, bottom-reading functionality is also required. See step 1.10.2 for an alternative to a plate reader for quantification of migrating cells.
4. **While cells are labeling, prepare the assay plate and lower chamber.**
   1. Use a 24-well tissue culture plate, with one condition per well. Perform each condition in triplicate.
   2. Add 750 µL of Hank's Buffered Salt Solution (HBSS) buffered with 25 mM HEPES and 0.1 % bovine serum albumin (BSA) to the 24-well plate. This serves as the basal control condition.
      1. Use other wells for comparator conditions, always maintaining the total volume in the bottom chamber at 750 µL.
         NOTE: In this example, human monocyte chemoattractant protein-1 (MCP-1) serves as the positive control for chemotaxis[7].
         1. Assemble the reagent mixture by combining HBSS with 25 mM HEPES, BSA (0.1 %), and MCP-1 (75 ng/mL). Use adult bovine serum (2.7 % v / v) with MCP-1 as an additional positive control.
            NOTE: Chemokine and chemokine concentration will depend on the chemotactic axis under study. Lyophilized MCP-1 is resuspended in distilled water with BSA (0.1%) to make a stock solution with a final concentration of 50 µg/mL. This can be diluted in water to a working solution of 10 µg/mL. Add 5.6 µL of this solution to the lower chamber.

   3. After the composition of the bottom chambers is complete, place a porous insert into each well, ensuring that the tabs of the insert align with the notches of the plate.
      NOTE: Choose the appropriate pore size for the inserts. For monocytes and lymphocytes, a pore size of 3 µm performs well. Some cell types and/or chemokine systems may require a matrix-coated surface for optimal migration. For example, for measuring THP-1 monocyte chemotaxis towards MCP-1, fibronectin or collagen-coated inserts are recommended. For plate reader for quantitation, the insert must have a light-impermeant coating, such as polyethylene terephthalate, to prevent detection of non-migrating cells in the upper chamber[8].
5. After incubating cells for 30 min with calcein-AM, transfer cell suspension to a 15mL conical tube. Centrifuge cells at 1,000 x g for 2 min. Visually inspect cells to confirm uptake of calcein-AM (**Figure 1**).
6. Carefully remove the supernatant using a vacuum aspirator. Wash labeled cells in serum-free RPMI 1640 supplemented with HEPES 25 mM. Centrifuge cells at 1,000 x g for 2 min and discard the supernatant.
7. Resuspend cells in serum-free RPMI 1640 supplemented with HEPES 25 mM. Count cells and adjust the volume so that the cells are at a final concentration of 1.0-1.5 x 10^6 cells/mL.
8. Dispense 250 µL of the cell suspension into the insert (upper chamber).
9. After all upper chambers have been filled, gently lift the plate, and inspect the underside for the presence of air bubbles that may interfere with migration and detection. To remove a bubble, first try removing then re-positioning the insert. If unsuccessful, use a 27 G needle to puncture the bubble and then re-position the insert.
10. **Place the plate into the pre-warmed 37 °C plate reader.**
    1. Acquire fluorescence readings from the bottom at 1-3 min intervals. When using calcein-AM, set the fluorescence excitation and emission wavelengths at 485 nm and 520 nm, respectively. Depending on the cell type and chemokine system, chemotaxis typically occurs over 1-4 h (**Figure 2A**).
    2. As an alternative to continuous measurements using a plate-reader, image the wells using an inverted fluorescent microscope (**Figure 2B**) capable of excitation and detection of light at 485 nm and 520 nm, respectively. Use a low magnification to capture the majority of the underside of the insert. Quantify cells by processing the images with ImageJ or similar software[9].

2. Adoptive Transfer of Murine Lymphocytes

1. **Preparation of donor cells**
   1. Anesthetize 8-12 week-old C57BL/6J male donor mice with isoflurane anesthesia (1-3 %) using a vaporizer. Confirm appropriate depth of anesthesia by toe-pinch.
   2. Place the mouse in the supine position. Make a midline incision with a scalpel and locate the spleen in the upper left peritoneal space. Excise the whole spleen and place into complete media (RPMI supplemented with 10% FBS and 25 mM HEPES; 1 mL/spleen) on ice. Euthanize anesthetized donor animals by decapitation.
   3. Grind spleen tissue gently through 40 µm nylon mesh into complete media (1 mL /spleen) using the end of a syringe plunger to make a single-cell suspension and transfer to a 15 mL conical tube.
   4. Add 4 volumes of ammonium chloride potassium (ACK) lysis buffer and incubate for 5 min at room temperature to lyse erythrocytes.
   5. Centrifuge the cells at 1,000 x g for 2 min at room temperature and discard supernatant. If erythrocytes remain in the pellet, resuspend in ACK lysis buffer, incubate for 5 min at room temperature, centrifuge cells at 1,000 x g for 2 min and discard supernatant.
   6. Resuspend the cells in complete media at a concentration of 2-5x10^7 cells/mL, then strain the cell suspension through a 40 µm nylon mesh to remove cell debris.
   7. Label 1 x 10^7 cells/recipient mouse with a fluorescent dye compatible with live cells and that will be retained upon subsequent fixation, such as 5-chloromethylfluorescein diacetate (final concentration 2 µM)[10]. Incubate at 37 °C for 30 min.
      NOTE: To track more than one cell population, use other fluorescent dyes with additional cell populations. For example, a portion of donor cells can be labeled with 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (final concentration 2 µM)[11].

8. Add 4 volumes HBSS supplemented with 25 mM HEPES, then centrifuge at 1,000 x g for 2 min and discard supernatant. Resuspend pellet in HBSS supplemented with HEPES 25 mM to a final concentration of 1 x 10^8 cells/mL.
    1. If using more than one fluorescent dye, keep cell populations separated until immediately prior to injection.
    2. Transfer a small aliquot (10 µL) of cells of each color to a fixative solution (1 mL) for subsequent flow cytometry compensation.

2. **Transfer of lymphocytes**
    1. Anesthetize 8-12 week-old C57BL/6J male recipient mice with isoflurane (1-3 %) using a vaporizer; confirm depth of anesthesia by toe-pinch. Apply veterinary ointment to animal's eyes to prevent drying.
    2. Briefly vortex donor cells (1 s) and transfer to 1 mL injection syringe with 27 G, 0.5 in needle, combining differentially labeled cell populations, if applicable.
        1. Transfer a small aliquot (10 µL) of mixed cells to a fixative solution (1 mL) for subsequent flow cytometry analyses.
    3. Place donor animals in the prone position. Apply mild caudal pressure to the skin dorsal to the eye, causing the eyeball to protrude slightly.
    4. Direct the needle medially and inject 100 µL of donor cell suspension retro-orbitally into each recipient mouse. Cells can also be injected via the tail vein.
    5. Gradually withdraw the injection needle and place mouse alone in a recovery cage. Monitor animal until it has regained consciousness; once fully recovered return the animal to social housing.

3. **Collection and Analysis**
    1. After 1 h, anesthetize recipient mice with isoflurane anesthesia (1-3%) using a vaporizer; confirm depth of anesthesia by toe-pinch. Harvest the spleen (or other tissue of interest) (step 2.1.2.) from each recipient mouse and place into complete media (1 mL/recipient). Euthanize anesthetized animals by decapitation.
    NOTE: Longer intervals prior to tissue harvest will increase tissue accumulation through at least 24 h.
    2. Grind spleen tissue gently through 40 µm nylon mesh into complete media using the end of a syringe plunger to make a single-cell suspension and transfer to 15 mL conical tube.
    3. Add 4 volumes of ACK lysis buffer and incubate 5 min at room temperature. Centrifuge the cell suspension at 1,000 x g for 2 min at room temperature and discard supernatant.
    4. Resuspend cells in staining buffer (phosphate-buffered saline supplemented with 1% FBS). Count cells and adjust to a final concentration of 3 x 10^7 cells/mL. Transfer 100 µL of cell suspension to a 96-well round bottom plate.
    5. Stain cells for desired markers (see the Table of Materials) for analysis by flow cytometry. Add fluorophore-conjugated antibodies (see the Table of Materials) against desired markers and incubate at 4 °C for 20 min in the dark. Add 100 µL staining buffer, centrifuge cells at 1,000 x g for 3 min and discard the supernatant.
    6. Resuspend the pellet in 200 µL staining buffer, centrifuge cells at 1,000 x g for 3 min and discard the supernatant.
    7. Resuspend the pellet in 125 µL staining buffer and acquire samples using a flow cytometer using standard procedures.
    NOTE: The green dye is excited by a 488 nm laser and the emission is detected with 505 and 530/30 dichroic filters. The orange dye is excited by a 561 nm and the emission is detected by a 582/15 dichroic filter.
        1. Use saved cells from 2.1.8.2 to compensate for each fluorescent dye.
        NOTE: Using bead-based compensation with fluorophores matching the excitation and emission spectra of the labeling dyes will result in suboptimal compensation.
        2. Use the saved input cells from step 2.2.2.1 to accurately determine the ratio of differentially labeled input cells if using more than one labeled population (**Figure 3**). Calculate tissue specific migration based on the ratio of labeled to unlabeled cells (**Figure 4**).

Representative Results

When using calcein-AM dye, visual inspection of cells will confirm label uptake (**Figure 1**). Automated fluorescent readings will track migration as cells transit onto the underside of the insert over time. These data clearly show an induction of cell migration towards MCP-1, as well as an augmentation of this response by serum (**Figure 2A**). Depending on the strength of the migratory stimulus, there is a lag of at least 15 min prior to an increase in signal. The fluorescent signal may peak and then gradually decline. This represents a net decrease in the number of cells adhering to the underside of the insert as cells pass into solution into the lower chamber at a rate faster than that of cells migrating from the upper chamber. Stronger migratory stimuli typically result in a) earlier onset of a rise in fluorescence values; b) faster rate of increase in fluorescence; and c) higher peak fluorescence values. Representative images acquired by inverted fluorescence microscopy are also shown (**Figure 2B**), with quantitation of cell counts (**Figure 2C**).

For *in vivo* studies utilizing more than one fluorescent label, it is important to quantitate the relative proportion of cell populations in the input material. This accounts for the variance in mixing cell populations for injection (**Figure 3**). In this case, the ratio of green- and orange-fluorescent cells was 0.97:1. Thus, to account for this slight imbalance, green-fluorescent cell counts were divided by 0.97 to index their numbers in proportion to the input material. After quantifying labeled cells by flow cytometry, trafficking can be quantified as the number of labeled cells recovered divided by the total number of cells recovered.

These results demonstrate the advantage of using the dual-color strategy, which eliminates the effect of varying injection efficacy; while there is considerable variance of recovered cells between animals, for any given animal the ratio of green-fluorescent to orange-fluorescent cells is approximately equal (**Figure 4**). These results are expected, as the two cell populations were treated identically, but various perturbations can be tested for their effect on lymphocyte homing. Deviation from the line of identity would indicate different migratory capabilities of the specific cell populations. Additionally, multiple cell subsets can be determined by staining the recovered cells for the desired proteins. Here, T-cells, indicated by CD3 staining, and B-cells, indicated by CD19 staining are also recovered with equal proportions of green- to orange-fluorescent cells.

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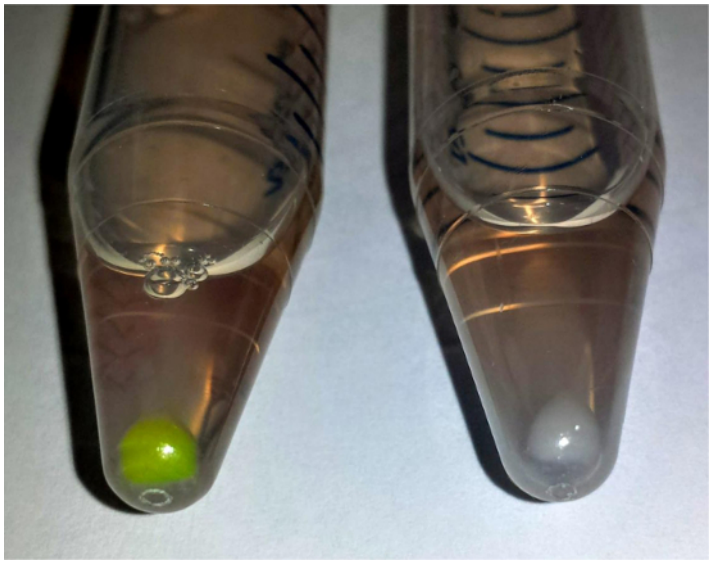

Figure 1: Cell labeling. Visual inspection of the cell pellet after labeling confirms adequate uptake of the fluorescent dye (left), in contrast to prior to labeling (right). Please click here to view a larger version of this figure.

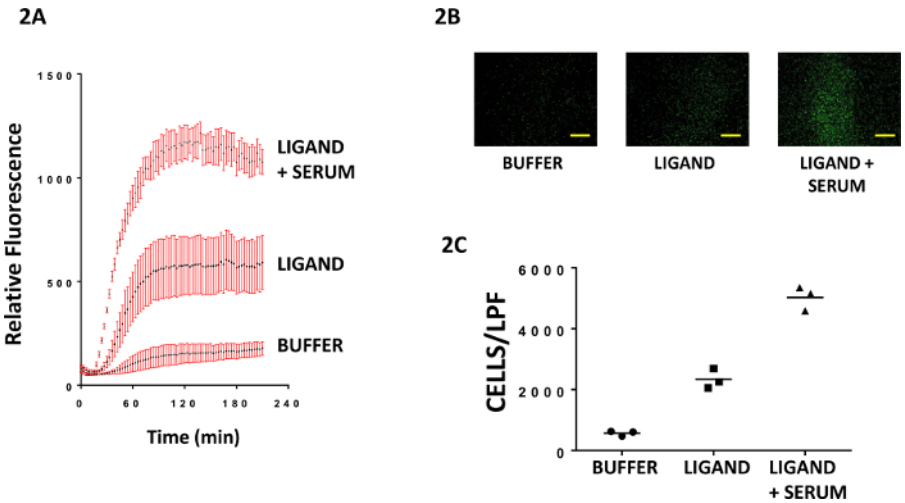

Figure 2: Quantification of *in vitro* Migration. (**A**) Representative data (mean ± standard error) acquired in real-time from a plate reader comparing 3 conditions: media (control), chemokine (MCP-1, 75 ng/mL) or chemokine with serum (MCP-1, 75 ng/mL and bovine serum, 2.7 % v/v in buffer). Readings were acquired from the underside of the plate every 3 min with an excitation wavelength of 485 nm and detection wavelength of 520 nm. (**B**) Representative micrographs at 4X magnification of the underside of the insert at 1 h, which allows direct visualization of migrating cells. Scale bars = 500 µm (yellow). (**C**) Quantification of cell number per low-powered field (LPF; 4X) using ImageJ software. Please click here to view a larger version of this figure.

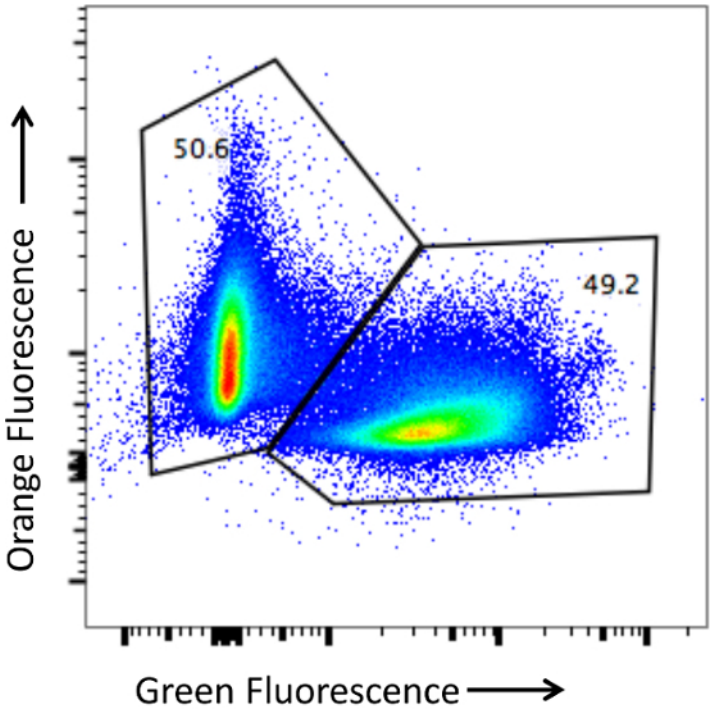

Figure 3: Stained input cells. Flow cytometry plot showing the ratio of the two differentially stained cell populations in the injected cell input from step 2.2.2.1. The ratio determined from this plot serves to correct for variance introduced during the initial mixing of the two populations and is used to adjust cell counts during downstream data analysis. Please click here to view a larger version of this figure.

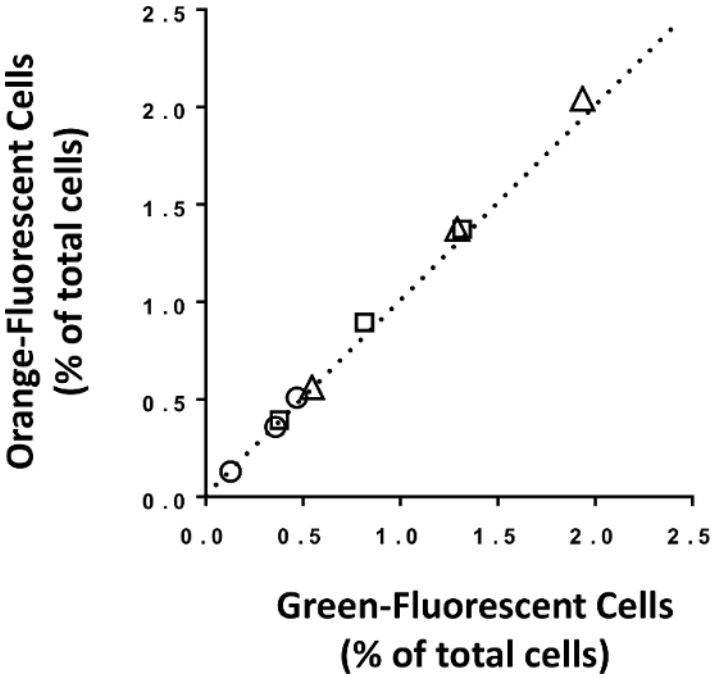

Figure 4: Ratio of labeled cells recovered from spleens of recipient animals. The proportions of green-fluorescent or orange-fluorescent cells are presented as a percentage of the total recovered splenocytes, with each data point representing a single recipient animal. The total labeled cells are shown (triangle), as well as the subsets that also stained positive for the cell surface markers CD19 (square) or CD3 (circle). Dashed line is the line of identity where the ratio between colors is equal to 1. Please click here to view a larger version of this figure.

Discussion

Quantification of immune cell migration can be accomplished using simple and rapid assays both *in vitro* and *in vivo*. We demonstrate the *in vitro* chemotaxis of human monocytes in response to a MCP-1 gradient and augmentation by serum. *In vivo*, donor murine splenocytes were differentially labeled and following adoptive transfer, were recovered from recipient animals.

Using a plate reader has the advantage of sampling several time points (as frequently as every 30 s) and automating the quantitation of migrating cells. This obviates the need to choose a single time point for evaluation and avoiding the more labor-intensive methods of manual counting of individual wells. Moreover, this method provides more dynamic information than can be gathered by microscopy. During analysis it must be kept in mind that cells will fall off the insert into the bottom chamber, and will not contribute to the fluorescence intensity. Thus, the number of cells on the underside of the insert, as indicated by relative fluorescence, will be a function of the rate of migration and the rate by which cells fall off the insert.

Larger cells may require an insert with larger pores, and 8 µm porous inserts are available. Smaller cells (including native lymphocytes) may not be bright enough to be reliably detected by a fluorimeter and may require evaluation by microscopy, particularly for small changes in chemotaxis. Some cell types may pass rapidly into the lower chamber solution and not have a long enough residence time on the insert to be detected, resulting in a flattened curve. In this case, cells can be quantified by cytometry directly from the lower chamber solution, or a matrix-coated insert can be used to lengthen transit time in addition to using an insert with smaller pores (*e.g.* 1 µm).

Critical to these assays is the identification of robust migratory conditions. For *in vitro* studies, the cell type studied must be capable of responding to a chemoattractant, and necessary co-factors must be considered. Optimization may be required for cell density introduced in the upper chamber. In general, a higher density will result in a stronger signal, but this needs to be balanced against a higher non-specific migration signal. Testing a range of chemoattractant concentrations may also help identify a suitable temporal window for capturing migration.

This method could also be adapted to use multiple colors. This would conceivably reduce variability, as well as increasing the number of conditions that can be included in a single assay. The primary consideration here is the brightness of the fluorescent dyes. Calcein-AM is very bright and therefore easily detected by both microscope and plate reader applications. Whereas the dyes used for *in vivo* studies were not readily detected by microscopy and produced high background; presumably this would also preclude accurate plate reader detection.

For *in vivo* studies, the health of donor cells is important to preserve their migratory function. The harvest of donor cells should be done as part of non-survival surgery, rather than sacrificing the donor animal and then harvesting cells, which increases the likelihood of ischemia and cell death. The time between injection and harvest is an important consideration for experiments in which different cell populations are used; the level of divergence or similarity between cell populations may change over time. Processing time after harvest from the donor until injection into the recipient should be minimized. Also, donor/recipient compatibility is also critical. Human cells will be rapidly rejected by mice, though intra-species allogeneic cells are well-tolerated over short periods. For example, we do not find a difference in migrating cells with C57BL/6J or BALB/cJ donors are used with C57BL/6J recipients over a period of 1 h.

This *in vivo* method is useful for assessing differences in migratory capability. Both input cells and recipient animals can be subject to pharmacological or other perturbations. Similarly, recipient animals can be exposed to infectious or inflammatory stimuli, or other treatments that affect lymphocyte trafficking[12]. This protocol can also be readily applied to transgenic mouse lines. In particular, those expressing fluorescent proteins would obviate the need for labeling and could potentiate multiplexing strategies.

It is important to note that the time in which different cell populations are mixed prior to injection should be reduced as much as possible. Differentially labeled cell populations should be mixed only after the recipient animals have been fully anesthetized and are ready for injection. The dyes present in the different populations can mix, resulting in unclear separation between the two when analysed by flow cytometry. In addition, different treatment conditions can affect the untreated cells during this time. For example, agents that bind to the cell surface may be transferred to the untreated cells, thereby confounding results.

Disclosures

The authors have nothing to disclose

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