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Techniques for Following Labeled Cells In Vivo: Use of X/Y FISH, Techniques to Optimize Fluorescent Detection, and Beta-Galactosidase Detection

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Abstract

The redistribution and trafficking patterns of cells to different anatomic sites throughout the body is important during cancer development and metastasis. Interest in the origin and fate of gastric cancer stem cells has recently arisen, as it may explain the underlying mechanism of cancer development. The ability to monitor the migration patterns of cancer stem cells is imperative to understanding the functional changes associated with the migration and proliferation of these cells. Here we detail a collection of techniques that include fluorescent in vivo imaging, X/Y FISH, and beta-galactosidase detection that are used for following labeled cells in vivo after adoptive transfer or transplant of donor cells for identifying the migration and engraftment of donor cells within the recipient.

Keywords

Gastric cancer; Stomach; Fluorescence membrane labeling; Inflammation; Cancer stem cells

1. Introduction

Methodologies enabling the trafficking of cells in vivo have made a tremendous impact on our understanding of the development of a number of gastric-related diseases including chronic inflammation and cancer. In particular, interest in the origin and fate of gastric cancer stem cells has recently arisen, as it may explain the underlying mechanism of cancer development (1–4). Cancer stem cell recruitment to the local tissue environment is a phenomenon that is related to the development of an inflammatory response in a variety of organs throughout the body (1, 3–7). In vivo approaches that investigators typically use to study migration and engraftment include implantation of cancer stem cells (either under the skin as a xenograft or “orthotopically” within the organ specific site) (8), adoptive transfer and bone marrow transplantation (1, 2). Determining the fate of cancer stem cells and understanding the functional changes associated with migration, engraftment, and proliferation require effective methods of identifying the in vivo trafficking pattern of these cells in the context of intact tissues and organ systems.

Understanding the origin and destination of gastric cancer stem cells and their link to the immune response advances our understanding of a variety of metastatic and inflammation-

induced cancers. Such knowledge would facilitate the development of novel therapies for the treatment of gastric cancer. Here we detail a collection of techniques that are used for following labeled cells in vivo after adoptive transfer or transplant of donor cells.

Techniques that include fluorescent in vivo imaging, X/Y fluorescence in situ hybridization (FISH), and beta-galactosidase detection have been shown to be valuable for identifying the migration and engraftment of donor cells within the recipient.

2. Materials

2.1. Fluorescence In Vivo Imaging of Cancer and Immune Cells

1. Phosphate buffered saline (PBS): For 1 l of 1× PBS: add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ to 800 ml of distilled water. Adjust the pH to 7.4 with HCl and add distilled water to a total volume of 1 l.
2. RPMI-1640 Medium: HyClone RPMI-1640 media (Thermo Scientific) with sodium bicarbonate, supplemented with 0.3 g per liter glutamine, endotoxin and cell culture-tested and filter-sterilized.
3. RPMI-1640/1% fetal bovine serum: RPMI-1640 medium supplemental with fetal bovine serum.
4. CellVue® Kits for Membrane Labeling: Purchased from Polysciences, Inc.
5. Red blood cell lysis buffer: In 800 ml distilled water add 8.3 g, 1.0 g KHCO₃ and 1.8 ml of 5% EDTA. Filter-sterilize through 0.2 µm filter and make up to 1,000 ml with distilled water.

2.2. Beta-Galactosidase Detection

1. Phosphate buffered saline (PBS): For 1 l of 1× PBS: add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ to 800 ml of distilled water. Adjust the pH to 7.4 with HCl and add distilled water to a total volume of 1 l.
2. 4% Paraformaldehyde: Measure 100 ml PBS into a glass beaker with a stir bar. Start stirring on hot plate IN THE HOOD. Set hot plate setting to about 60°C while making sure the solution does NOT boil. Add 4 g of paraformaldehyde. Continue to heat until the liquid turns from cloudy to clear. The pH may need to be raised until the solution clears. This can be achieved by adding a few drops (2–3) of 1 N NaOH. When the solution is clear, the pH is corrected with HCl. Check the pH by pipetting a small amount of the solution onto a pH strip. If the pH is greater than 8 or below 7 keep titrating. DO NOT titrate on the pH meter as this will be damaged by the paraformaldehyde. Turn off heat and continue to stir until cool. Store at 4°C. Alternatively, ampoules of premade 10% paraformaldehyde may be purchased and diluted using 10 ml of 10× PBS, 80 ml of distilled water and 10 ml of paraformaldehyde from a freshly opened ampoule.

3. Rinse buffer: 0.1 M Na phosphate (pH 8.3–8.5), 2 mM MgCl₂, 0.1% Na deoxycholate, and 0.2% Triton X-100 (or Nonidet P-40).
4. X-gal staining solution: To 100 mg X-gal (Sigma Aldrich, B4252, 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside) add 4 ml of *n*-dimethylformamide to make 25 mg/ml X-gal. Make stock solutions of the following:

0.25 M Potassium ferricyanide (FW 329.26, K₃Fe(CN)₆): 8.23 g in 100 ml distilled water.

0.25 M Potassium ferrocyanide (FW 422.39, K₄Fe(CN)₆): 10.56 g in 100 ml distilled water.

To the 4 ml 25 mg/ml X-gal add 2 ml Potassium ferricyanide and 2 ml Potassium ferrocyanide and bring to 100 ml volume with the rinse buffer. Mix and use immediately.

2.3. Alternate Protocol to Fixing Tissue—Paraformaldehyde Perfusion

1. 1× PBS.
2. 4% paraformaldehyde, freshly prepared and chilled at 4°C.
3. Two syringes (20 ml) with 23 gauge needles.
4. Scissors, scalpel, forceps.

2.4. X/Y FISH

1. 20× Saline–sodium citrate (SSC) buffer: 173 g NaCl (3 M), 88.2 g (300 mM) sodium citrate. Adjust the pH to 7.0 with a few drops of 14 N solution of HCl. Make up to 1 l with water.
2. Hybridization buffer: 50% v/v formamide, 10% dextran sulfate, 2× SSC, and 0.5 M phosphate buffer pH 7.3.
3. Denaturing solution: 70% v/v formamide, 2× SSC. Adjust pH to 7.0.
4. Carnoy's fixative: 60 ml ethyl alcohol, 30 ml chloroform, and 10 ml acetic acid.

3. Methods

3.1. Fluorescence In Vivo Imaging of Cancer and Immune Cells

The following protocol has been optimized to follow cell migration in response to *H. pylori* infection in the stomach. The images shown in Fig. 1 demonstrate migration of splenocytes in vivo in response to *H. pylori* infection (see Note 1). Here we present a protocol to that may be used to record the trafficking pattern of immune cells to improve our understanding of how the immune response is initiated. This protocol may be modified to track the

¹When this protocol is used with cancer cells the fluorescence of the dye starts to diminish 30 days post-transplant.

migration pattern of cancer cells in response to *H. pylori* infection (see Note 1). All animal work must be done under IACUC approval.

1. Remove spleen from a wild type uninfected mouse and store in ice-cold RPMI medium until homogenization.
2. Homogenize the spleen in 2 ml of RPMI medium and filter cells through a 40 μ m mesh into a 50 ml conical tube.
3. Centrifuge cells at $270 \times g$ for 10 min and lyse red blood cells by incubating cells in red blood cell lysis buffer (5 ml) for 5 min (see Note 2).
4. Add 20 ml of PBS to stop lysis.
5. Centrifuge cells at $270 \times g$ for 10 min and aliquot 1×10^8 cells in 5 ml PBS for fluorescence labeling.
6. Prepare a working dye stock of CellVue Maroon by adding 20 μ l of dye in 5 ml of diluent C (provided by the company) and add to 5 ml cell suspension.
7. Incubate for 5 min at room temperature.
8. Add 10 ml RPMI/1% FBS to stop the reaction.
9. Centrifuge cells at $270 \times g$ for 10 min.
10. Wash cell pellet three times with 10 ml RPMI/1% FBS.
11. Centrifuge cells at $270 \times g$ for 10 min and resuspend cells in sterile PBS at a concentration of 2×10^6 cells/200 μ l/mouse.
12. Splenocytes isolated from an uninfected mouse are injected intraperitoneal into an *H. pylori* infected host mice using a 25 gauge needle and 1 ml syringe.
13. Mice are anesthetized using isoflurane, and imaged on the In Vivo Multispectral Imaging System (Carestream Molecular Imaging, CMI) with exposure time 10 s (excitation filter 630 nm, emission filter 700 nm) and subjected to X-ray (35 KVP, 30 s exposure, 0.5 mm filter) (Fig. 1) (see Note 3). Mice are imaged every day up to 7 days post-adoptive transfer.

3.2. Beta-Galactosidase Detection

Genetic labeling of cells using the *Escherichia coli* beta-galactosidase gene (LacZ) is an accepted method used for the identification, localization, and engraftment of transplanted cells in vivo. Stable expression of beta-galactosidase expression within cells may be detected using a sensitive immunohistochemical system for detecting LacZ. The protocol for beta-galactosidase staining was modified from a published method by Hendrikx et al. (9)

²With incubation in red blood cell lysis buffer the solution should appear clear when red blood cells are lysed.

³Mice may be imaged over several days by shaving the abdomen and for the final imaging, after euthanasia, body cavity can be opened for anatomical localization of fluorescence (Fig. 1.).

1. Collect stomach tissue and wash well in 1× PBS.
2. Pin tissue to a piece of dental wax placed in a well of a six well plate (see Note 4). Fix stomach tissue with 4% paraformaldehyde for 1 h on ice.
3. Wash tissue using rinse buffer for 30 min three times at room temperature.
4. Stain tissue from 4 h to overnight with X-gal staining solution at 37°C in the dark (see Note 5).
5. Wash tissue with rinse buffer for 30 min at room temperature.
6. Post-fix tissue overnight in 10% formalin at 4°C (see Note 6).
7. Paraffin-embed tissue and section (see Note 7).

3.3. Alternative Protocol for Fixing Tissue: Paraformaldehyde Perfusion

For detecting transplanted cells with a higher sensitivity while maintaining good gastric morphology and preserving other organs, perfusion of the mouse with a fixative for in vivo fixation may be necessary. The protocol detailed here is for mice and is based on a published protocol by Zeller (10). The method does not require a pressure-controlled perfusion pump and involves the exchange of the blood with PBS and then with paraformaldehyde (10).

1. Fill one syringe with 20 cm³ 1× PBS, and another with 20 cm³ 4% paraformaldehyde and store at 4°C on ice. The syringes will be attached to a 25 gauge butterfly needle with a stopcock. This will allow syringes to be changed without the need for a second stick.
2. Euthanize mouse by CO₂ inhalation under approved protocol and immediately lay animal on its back. DO NOT euthanize by cervical dislocation.
3. Pin the mouse to a Styrofoam board—using push pins or 18 gauge needles inserted through the paws.
4. Make a midline incision through the skin, and the underlying abdominal muscle to expose the peritoneal lining.
5. Carefully open the peritoneal lining being cautious not to nick the bowel. Pin the skin, muscle, and the lining to a Styrofoam board. This will immobilize the mouse and allow an easily viewed field.
6. Carefully cut through the rib cage and remove the diaphragm to access the heart. It is advised to work quickly but carefully. If the blood clots or the main blood vessels are damaged the perfusion cannot be performed.

⁴Stomach sections may be pinned onto a piece of dental wax to keep tissues flat during the fixing and staining procedure.

⁵The tissue color may be monitored over this period of time. For strong signals tissues will become blue within 4 h of staining with X-gal solution. Staining may take up to 24 h. In our experience, any staining that appears after 24 h is nonspecific and should be interpreted cautiously. Always interpret in the context of the appropriate negative control. Tissue collected from nontransplanted mice may be used as a negative control.

⁶After fixing overnight in 10% formalin, tissue may be rinsed in PBS and stored in 70% ethanol at 4°C until paraffin-embedded.

⁷After sectioning, tissue may be counterstained by either immunohistochemistry or immunofluorescence.

7. Cut the right atria to allow blood to flow out.
8. Carefully insert the needle attached to the syringe filled with 1× PBS, into the left ventricle.
9. Slowly but constantly perfuse the 1× PBS into the heart (Fig. 2). You will know if the perfusion is working if the liver and spleen turn grayish-white.
10. After the blood has been flushed out, remove the syringe with 1× PBS and attach syringe filled with ice cold 4% paraformaldehyde. It is often helpful to have a second person help you with this step. Slowly perfuse the mouse with 20 ml paraformaldehyde. A successful perfusion is indicated by a muscle tremor that is observed on the limbs and tail. By the end of this procedure the animal should be stiff.
11. Following the perfusion, dissect out stomach and rinse out contents with PBS.
12. Proceed with steps 3–7 outlined above for the beta-galactosidase detection.
13. If direct fluorescent imaging is indicated, the tissue is processed in OTC for frozen section. Alternately, tissue can be processed for routine histology.

3.4. X/Y FISH

The following method was modified using the package insert for the CEP X Spectrum Orange/Y Spectrum Green DNA Probe Kit as a guide (Abbot Molecular Inc., Product Number 30-161050/32-161050).

3.4.1. X/Y Paint Production—A variety of commercially available paints are available for X/Y-FISH. The Vysis CEP X/Y probe set (Abbott Molecular, Inc.) targets centromeric alpha-satellite Xp11.1-q11.1 and satellite III Yq12 loci yielding orange and green fluorescence, respectively. If commercial chromosome paints are not available for the sample being evaluated, then the following steps should be used to generate the necessary paints:

Isolate chromosomal material from the tissue sample or cell population of interest (using standard method for the chosen cell type). An example of such a purification may be found in Rens et al. (11).

1. Prepare the chromosomes for sorting by staining with 40 µg/ml of Chromomycin A3 (Sigma), 2 mM MgSO₄, and 2 µg/ml Hoechst 33258 for at least 2 h.
2. Immediately prior to sorting, add 10 mM sodium sulfite and 25 mM sodium citrate.
3. Collect X and Y chromosomal material in separate PCR tubes containing 30 µl of sterile water.

4. Use the chromosomes as a template for degenerate oligonucleotide primer (DOP) PCR.
5. Perform DOP-PCR using labeled nucleotides in order to generate “painted” product DNA. Biotin-16-dUTP and Cy3-dUTP are recommended.
6. Dilute the X and Y paints to 50 ng per 15 µl of hybridization buffer.

3.4.2. Interphase/Metaphase Cell Preparation—Isolation of interphase or metaphase cell populations from sex-mismatched, bone marrow transplanted animals should be performed according to the AGT Cytogenetics Laboratory Manual (12).

1. Collect bone marrow samples in sodium-heparinized vacutainers and use immediately to prepare slides. Hemolyzed samples should not be used for FISH analysis.
2. Prewarm denaturing solution to 73°C.
3. Wash twice in iced PBS.
4. Resuspend 10^5 cells in 0.2 ml of ice cold PBS. (Note: A minimum of 100 µl is required for cytopsin slide preparation, with the overage included if a concentration adjustment is needed to achieve the desired number of cells per image field. This volume should be scaled based on the number of replicates required.)
5. Prepare a cytopsin device by placing slides and filters into the appropriate slots. The cardboard filter should face the center of the cytopsin. Prewet each well with 100 µl of cold PBS and spin for 1–2 min.
6. Quickly add 100 µl (approximately 5,000 cells) of each sample to the appropriate wells of the cytopsin and spin for 5 min at 500 rpm. Carefully remove the filters and slides being careful not to disrupt the pelleted cells.
7. Visually examine each slide using low power phase contrast microscopy (10× magnification) in order to confirm that the cells have pelleted properly and that each field contains at least 100 interphase cells. Air-dry slides.
8. Fix slides in Carnoy’s fixative for 15 min and air dry.
9. Hold the prepared slides in a dark slide box at –20°C until needed for hybridization.

3.5. Fluorescence In Situ Hybridization Assay

3.5.1. Denaturation of Specimen DNA

1. Denature the specimen DNA by submerging the slides in 73°C denaturing solution for 5 min.
2. Remove slides from bath.

3. Dehydrate slides through a 1 min transfer series of 70, 95, and 100% ethanol baths.
4. Prepare all probe hybridization solutions and allow the X,Y paint probes to warm to room temperature.
5. Immediately before hybridization, drain the excess alcohol by wicking solution from the edge of the slide onto a paper towel.
6. Dry the slides on a 50°C slide warmer for 2 min and proceed immediately to the next step.

3.5.2. Hybridization

1. Vortex probe solution to mix. Denature mixture in a 75°C water bath for 5 min.
2. Obtain the desired test slides and appropriate low proportion male (5% XY and 95% XX) and low level female (5% XX and 95% XY) control smears.
3. Add 10 µl of the probe solution and 10 µl of hybridization buffer to each cytospin and coverslip. Air bubbles trapped under the coverslip interfere with hybridization and should be avoided.
4. Transfer slide(s) to a prewarmed, covered chamber and transfer to the hybridization oven. Hybridization may be performed for a minimum of 30 min at 42°C or, if preferred, overnight at 37°C (see Note 8).
5. Following hybridization, slides may be sealed (around the edge of the coverslip) with rubber cement and held in a humidified hybridization chamber overnight (<16 h) until final washes are performed.
6. Carefully remove the coverslips and wash for 5 min in 50°C 1× SSC buffer (see Note 9).
7. Transfer slides to 0.5× SSC for 5 min.
8. Quickly rinse slides in distilled water, wick off excess liquid, and air dry in the dark.
9. Coverslip and seal the slides using 20 µl of DAPI II mountant.
10. Store hybridized slides desiccated at –20°C for up to 1 year.

3.5.3. Analysis: Quality Verification

1. Scan slides under wide-field fluorescence using a 25× objective to confirm uniform, bright signal intensity, dark background intensity, and specificity of hybridization. Acceptable staining can present as either compact, brightly stained points or more dimly fluorescent, diffuse oval-shaped

⁸Hybridization may be performed for a minimum of 30 min; however, to increase the signal intensity, hybridization time may be increased to overnight.

⁹For an alternative approach using less stringent 0.4× SSC, wash at elevated 73°C temperature.

points. Elevated background or reduced signal-to-noise intensities may result from insufficient washing, hybridization stringency, or improperly high paint concentration. Additionally, probes may crosshybridize to nontarget sequences if stringency is too low. If either of these situations occur, optimization of these parameters should be performed with positive and negative control slides used for each set of assay conditions.

2. A majority (>95%) of cells should display one or both of the *fluorescent signals*.

3.5.4. Analysis: Control Slide Requirements

1. View each control slide through a 25× or 63× objective. Image cells using fluorescence filter sets appropriate for the chosen X and Y paints or according to manufacturer instructions. (Imaging each emission channel sequentially will help to identify areas of signal overlap between fluorophores.)
2. Choose an area of the cell smear that is uniformly distributed with minimal cell overlap and clumping.
3. Count the number of signals from a total of 500 interphase nuclei and 20 metaphase spreads beginning in the upper left quadrant image field and scanning from left to right, top to bottom. Only *distinct* signals should be counted from interphase nuclei, and only nonoverlapping chromosomes with clear staining should be counted for metaphase spreads.
4. The assay should be repeated if less than 95% of the nuclei show specific staining.
5. Tally the number and percentage of interphase nuclei with XX and XY signals. Greater than 95% of the cells should be clearly identified as either XX or XY. The percentage of XX and XY cells should fall within the data sheets provided with the control slides.

3.5.5. Analysis: Sample Interpretation

1. Enumerate test slides as described above for the control slides.
2. Specimens with greater than 0.6% of the interphase cells identified as the donor gender are considered positive for the presence of donor cells (e.g., 3 out of 500 total cells). Similarly, if any of the 20–30 metaphase spreads are identified as having donor origin, the specimen is considered positive.

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Fig. 1.

Fluorescence in vivo imaging of labeled splenocytes 7 days post-adoptively transferred into *H. pylori*-infected mouse. The images shown are of a mouse closed (*left image*), opened (*middle image*) and dissected organs (*right image*) showing expression of labeled splenocytes within the stomach at the site of infection (also see Note 3). Mice may be imaged over several days by shaving the abdomen. The body cavity can be opened for anatomical localization of fluorescence (Fig. 1). *ST* stomach, *LIV* liver, *SP* spleen.

Fig. 2. Paraformaldehyde perfusion of the mouse. The diagram and photo illustrates the chambers of the heart and the positioning of the syringe in the left ventricle during perfusion.