Migration of Bone Marrow Stromal Cells in 3D: 4 Color Methodology Reveals Spatially and Temporally Coordinated Events

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The cytoskeleton plays a central role in many cell processes including directed cell migration. Since most previous work has investigated cell migration in two dimensions (2D), new methods are required to study movement in three dimensions (3D) while preserving 3D structure of the cytoskeleton. Most previous studies have labeled two cytoskeletal networks simultaneously, impeding an appreciation of their complex and dynamic interconnections. Here we report the development of a 4 color method to simultaneously image vimentin, actin, tubulin and the nucleus for high-resolution confocal microscopy of bone-marrow stromal cells (BMSCs) migrating through a porous membrane. Several methods were tested for structural preservation and labeling intensity resulting in identification of an optimized simultaneous fixation and permeabilization method using glutaraldehyde, paraformaldehyde and Triton X-100 followed by a quadruple fluorescent labeling method. This procedure was then applied at a sequence of time points to migrating cells, allowing temporal progression of migration to be assessed by visualizing all three networks plus the nucleus, providing new insights into 3D directed cell migration including processes such as leading edge structure, cytoskeletal distribution and nucleokinesis. Colocalization of actin and microtubules with distinct spatial arrangements at the cellular leading edge during migration, together with microtubule axial polarization supports recent reports indicating the pivotal role of microtubules in directed cell migration. This study also provides a foundation for 3D migration studies versus 2D studies, providing precise and robust methods to attain new insights into the cellular mechanisms of motility. Cell Motil. Cytoskeleton 63:725–740, 2006.

Key words: bone marrow stromal cells; quadruple labeling; cytoskeleton; confocal microscopy; multiphoton microscopy; chemotaxis; cell motility; cell migration

INTRODUCTION

The use of cell-based therapies, biomaterials and tissue engineering for repair and regeneration of tissues such as cartilage, are expected to provide efficacious alternatives to existing therapies. Improved understanding of the mechanisms underlying the multiple roles of cells involved in tissue engineering and wound healing, including migration, wound contraction and response to

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mechanical loads, requires robust methods to illuminate the underlying cytoskeletal structures and functions in these processes. The mammalian cell cytoskeleton is mainly composed of microfilaments (MF) and microtubules (MT), both of which are highly conserved, in addition to intermediate filaments (IF), which are cell and tissue specific, with vimentin related to fibroblast-like phenotypes [Helfand et al., 2004]. These networks are responsible for cellular spatial organization and are associated with a wide array of proteins which impart functional diversity and permit cell locomotion [Rozycki et al., 1994; Chang and Goldman 2004].

Cytoskeletal filaments are formed by polymerization of monomers and are highly dynamic and labile structures sensitive to physico-chemical environmental changes [Hibbs, 2000]. In addition, each network is in the presence of its monomers in the cytoplasm and is partly masked by a variety of associated soluble and insoluble proteins which can complicate immunocytochemistry procedures [Arcangeletti et al., 1997]. Limiting factors for 3D resolution of cytoskeletal elements in immunofluorescence lies in the concurrent achievement of good structural preservation and detailed labeling [Robinson and Snyder, 2004]. Most cytological processing methods are not optimized for 3D cell preservation, and therefore do not harness the full potential of confocal microscopy to assess cell structures representative of the physiological steady state [Arcangeletti et al., 1997]. To date, structural work in this area has mostly involved the labeling of two cytoskeletal networks in single cells, with important exceptions of triple immunofluorescence [Herzog et al., 1994; Baschong et al., 1999] but without quadruple labeling including the nucleus. These elements are therefore usually observed as separate entities or in pairs, when in fact they form a complex interwoven system [Chang and Goldman, 2004], where cytoskeletal crosstalk is now deemed essential, particularly between MT and MF [Small et al., 1999a; Rodriguez et al., 2003; Etienne-Manneville and Hall, 2003] and to a lesser extent with IF [Goldman et al., 1999]. The complexities involved in imaging moving cells in 3D matrices has also resulted in most information related to the cytoskeleton in cell migration being gained from in vitro studies carried out in two dimensions [Palecek and Ubbels, 1997]. Although it is believed that mechanistic understanding of migration along a 2D surface is relevant to 3D movement [Lauffenburger and Horwitz, 1996], some studies in 3D gel matrices have demonstrated significant differences in cell morphologies as well as in spatial organization and molecular composition of adhesions complexes [Cukierman et al., 2001; Grinnell, 2003], and hence, different migration behavior [Friedl and Brocker, 2000; Wolf et al., 2003]. Unfortunately there are no reports to date of simultaneous visualization of all major cytoskeleton networks and nucleus in 3D, in a controlled directed-migration system.

In this report we describe a method using four fluorescent stains to simultaneously label the main cytoskeletal systems of vertebrate cells, namely vimentin, tubulin and actin in addition to the nucleus for confocal 3D visualization of the cytoskeleton of rabbit bone marrow stromal cells (BMSCs) migrating through a porous 10 µm thick polycarbonate membrane. The quadruple labeling combines a direct antibody (Cy3-anti-vimentin) and a labeling with a secondary anti-body (anti-tubulin) with two intercalating fluorescent markers (Alexa 488-phalloidin for actin and TOTO for DNA) resulting in a strategy that minimizes background fluorescence to a degree which would be difficult to achieve using primary and secondary antibody labeling alone. This multilabeling study, based on a previous method for chondrocytes in culture by Blanc et al. [2005], has been adapted to BMSC migrating through a porous membrane and provides a 3D model close to physiological reality while still allowing distinct visualization of the complex interplay between cytoskeletal elements involved in migration. We have tested numerous protocols in order to achieve optimal cell structure preservation in 3D together with ideal labeling intensity and fine detail resolution by examining fixative type and detergent concentrations, temperature, pH and physical manipulation, all of which can influence cytoskeleton network structure, in addition to the sensitivity and selectivity of antibody-antigen reactions [Larsson, 1998].

To obtain optimal preservation and structural imaging, we tested several fixatives including precipitating agents and the crosslinking agents paraformaldehyde (PFA) and glutaraldehyde (GA). Permeabilization with detergents Triton X-100 or Octyl-Poe at varying concentration was also performed either before, simultaneously or after fixation. Precipitating agents serve here as “negative” controls since they are known to flatten samples, thereby limiting out of focus light, which can be seen as beneficial in projected images of non-confocal microscopy. PFA has a relatively slow rate of crosslinking compared to GA, but is 10 times faster in diffusive penetration rate [Hopwood, 1967] and can therefore be useful in preserving physiological cell structure [Bacallao et al., 1995]. Although highly labile elements such as tubulin are not well preserved with PFA [Palecek and Ubbels, 1997], we have included it based on its wide application, as in the few previously applied 3D methods [Bacallao et al., 1995]. The slower penetration rate of GA compared to PFA is compensated by its faster crosslinking and its remarkable preservation capabilities for subcellular organelles [Hibbs, 2000]. However, GA increases autofluorescence, and has the ability to change protein
tertiary structures, and hence to destroy or mask epitopes and limit antibody accessibility [Larsson, 1988]. No single fixative has emerged as an ideal fixative for preserving all three networks in all conditions and cell types so that studies that examine these parameters such as ours are essential.

Here we focus on BMSCs, in a rabbit model, based on their role in cartilage regeneration therapies. Recent work by our group has identified an implantable biomaterial that is capable of increasing recruitment of BMSCs from bone marrow to cartilage lesions to stimulate repair [Hoemann et al., 2005; Chevrier et al., in press]. BMSCs can also be induced to express markers of various connective tissues making them well suited for cell therapy in regenerative medicine [Shapiro et al., 1993]. Despite the recent interest in BMSCs and the large scale use of rabbit models in joint related disorders studies [Rudert, 2002], there is a paucity of functional cell studies such as in vitro chemotaxis and relevant chemotactic agents for this specific cell type. We found recombinant human platelet derived growth factor (PDGF) at relative high concentration to be a potent chemoattractant for BMSCs, and we have elaborated a robust method that permits the simultaneous and precise labeling of the major cytoskeleton elements of BMSCs, while preserving their 3D structural integrity in transmembrane directed cell migration experiments.

**MATERIALS AND METHODS**

**Cell Isolation and Sample Preparation**

Femurs from New Zealand White Rabbits \( (N = 5) \) weighing between 3 and 4 kg were used. To isolate femurs, both legs were first shaved from the tarsal joint to the hip using an Oster Animal Clipper with a size 40 blade set. Femurs were cut from the joint and placed in sterile HBSS-FBS (Hanks Buffered Salt Solution from Invitrogen, Carlsbad, CA supplemented with HEPES 10 mM + 2% FBS + 100 units Penicillin and 0.1 mg streptomycin /ml) on ice. The femurs were then cut open at both epiphysis with a bone cutter and flushed with 10 ml syringes containing HBSS-FBS into a 100 mm Petri dish. A dispersed bone marrow cell suspension was then prepared by repeated pipetting of the cell suspension with decreasing size needles, from 18 to 23 g and then prepared by repeated pipetting of the cell suspension filtered through a 70 \( \mu \)m mesh to remove remaining particulates of fat or bone and centrifuged and washed two times in HBSS-FBS. Erythrocytes were lysed in a 1 ml aliquot with 0.14 M ammonium chloride and remaining cells counted in a hemocytometer and seeded at \( 10^7 \) cells per 100 mm Petri dish and adherence purified for propagation in 25 cm\(^2\) culture flasks for colony forming efficiency (CFE) analysis. CFE was estimated by fixing cells in the flask at day 14 using methanol for 10 min, staining with 1% w/v toluidine blue in PBS (phosphate buffered saline, Sigma, St. Louis, MO) for 5 min and counting colonies under a dissecting microscope (data not shown). Culture media was DMEM low glucose (Invitrogen, Carlsbad, CA) supplemented with fresh 10% FBS (Sigma, St-Louis, MO) and 100 units Penicillin and 0.1 mg streptomycin /ml (Sigma, St. Louis, MO).

**Cytoskeleton Imaging on 2D Surfaces**

All samples that were immunolabelled, with the exception of those used in migration studies described below, were cultured on no. 1 sterile 12 mm diameter round coverslips (EMS, Hatfield, PA). Coverslips were sterilized by ethanol soaking and ultraviolet light drying and placed in 24 well plates on which cells were seeded at a density of 40,000 cells per ml in 500 \( \mu \)l per coverslip and incubated for at least 24 hr to ensure complete adherence and cytoskeleton stabilization prior to processing.

**Permeabilization and Fixation**

For all conditions below, prior to processing cells, culture medium was aspirated and cells washed 3 \( \times \) 5 min in mHBSS (136.9 mM NaCl, 5.36 mM KCl, 0.44 mM KH\(_2\)PO\(_4\), 0.336 mM NaHPO\(_4\), 2 mM MgCl\(_2\) 6H\(_2\)O, 4 mM NaHCO\(_3\), 2 mM EGTA, 5.5 mM d-Glucose and 0.11% w/v 2-morpholinoethanesulfonic acid (MES), pH \( \sim 6.5 \). Subsequent permeabilization and fixation (perm/fix) were also performed in this buffer, unless otherwise stated, since the low pH, calcium chelation and presence of magnesium is known to aid in the cytoskeletal stabilization and preservation [Bacallao et al., 1995]. As outlined in Table I, procedures are divided in 3 classes according to the fixative agents used, precipitating agents, PFA (Sigma, St-Louis, MO) or GA and the sequence of permeabilization and fixation. Concentrations below are expressed as %w/v for PFA and %v/v for GA, Triton X-100 and Octyl Poe.

**Precipitating Agents (Conditions 1 and 2 in Table I).**

Cells are either fixed in an ice-cold mix of equal volume of methanol and acetone or in ice-cold methanol alone for 6 min.

**Paraformaldehyde (Conditions 3 to 6 in Table I).**

In conditions using PFA as the fixation agent, permeabilization is either performed before, during or after fixation. Only freshly prepared PFA was used. In the standard condition, cells are fixed in a 4% PFA solution in mHBSS for 20 min at 37°C prior to permeabilization in a 0.5% Triton X-100 solution in mHBSS for 15 min. In the delayed fixation, the sequence is inverted, and cells are permeabilized first in a less concentrated 0.3%
Triton X-100 solution before fixation in 4% PFA at 37°C for 10 min. Finally, in the instantaneous method [Arcangeletti et al., 1997], perm/fix are performed simultaneously in one step, with a 0.5% Triton X-100/1% PFA buffer for 10 min at 37°C. The concentration of Triton X-100 was optimized, as suggested previously [Arcangeletti et al., 1997] using several concentrations-0.5, 1, 2.5, 5, 10, and 20% v/v where good results were obtained with all concentrations at and below 5% (data not shown). Only the lower concentrations, namely 0.5 and 1%, were examined in multiple stains, since their gentler effects on cells may be important for experiments on polycarbonate membranes, a substrate towards which cells exhibit reduced adhesiveness, compared to glass, that is necessary for migration.

**Glutaraldehyde (Conditions 7–13 in Table I).** The permeabilization detergent in procedures using GA as fixative is also added either before or during the fixation. For smooth fixation, based on the procedure of Small et al. [1986] and later from Baschong et al. [1999] who replaced detergent Triton X-100 by Octyl-Poe, cells were permeabilized in mild non-ionic detergent in mHBSS in the presence of a very low concentration of GA acting as a prefixation to stabilize the cell structure. Detergents used were either Octyl-Poe or Triton X-100, both at 0.5 and 2%. All 4 conditions incorporate a GA concentration of 0.125% during permeabilization, for 10 min at 37°C that is then followed by a 20 min fixation in 1% GA mHBSS at room temperature. For the instantaneous method, cells were also perm-fixed with a mix of low detergent and low GA concentrations, but without any post fixation. Kpipes buffer in the original article [Bacallao et al., 1995] was replaced by mHBSS, as the latter is routinely used with musculoskeletal type cells as opposed to endothelial cells for the former. Cells were perm/fixxed for 10 min with 0.3% GA + 0.1% Octyl Poe or Triton X-100 at room temperature. A second mix of 0.5% GA + 0.3% Octyl Poe or Triton X-100 was also tested. This latter method was supplemented with 0.3% PFA to constitute the final optimized processing method (condition 13 in Table I) allowing the procedure to profit from the rapid penetration of PFA and the fast crosslinking of GA.

**Post-Fixation Treatment.** Following perm/fix, cells from all conditions were washed 3 × 5 min in mHBSS. For cells treated with precipitating agents or PFA based fixatives, cells were then incubated for 20 min in 1% Bovine serum albumin (BSA) (Sigma, St. Louis, MO) to quench aldehyde residues [Arcangeletti et al., 1997].

Two methods using three different concentrations of sodium borohydride (NaBH₄) (Sigma, St. Louis, MO) were applied to identify a reliable method to remove autofluorescence induced by GA based fixations. For GA fixed samples, the method we evaluated (data not shown) to be most effective in quenching background fluorescence, was 0.5 mg/ml NaBH₄ in ice cold buffer incubated for 2 ×

<table>
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<td>Instantaneous PFA perm/fix 2</td>
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<td>0.5% Octyl Poe/0.125% GA, 5 min</td>
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<tr>
<td>Smooth GA perm/fix 4</td>
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<td>Mix of PFA/GA</td>
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<tr>
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<td>13</td>
<td>0.5% GA/0.3% PFA/0.3% Triton X-100, 20 min</td>
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% expressed as w/v for PFA and v/v for GA, Triton X-100 and Octyl Poe.
10 min on ice and was used subsequently in these studies. Cells were washed 3 × 5 min afterwards in PBS. Before labeling, non specific binding sites were blocked by incubation in 10% goat serum solution in mHBSS added with 0.05% Tween-20 (Sigma, St. Louis, MO) for 30 min.

Fluorescence Labeling

**Triple Stain.** The sequence of labeling for multiple stains can have a dramatic effect on image quality due to cross reactions and steric hindrance. Indirect labeling using secondary antibodies should precede direct labeling with conjugated antibodies or specific toxins such as phalloidin, and polyclonal antibodies should be used prior to monoclonals [Baschong et al., 1999]. After one cell wash in mHBSS-GST (mHBSS supplemented with 1% Goat serum and 0.05% Tween-20), cells were labeled first with 2.5 μg/ml (1:400 dilution) anti-vimentin Cye 3 conjugated monoclonal antibody (Sigma, St. Louis, MO) in mHBSS-GST with 400 ng/ml of DiI live-cell stain and compared to cell height after fixation that was measured from X-Z and Y-Z sections of 3D stacks of randomly selected cells using built-in functions in the LSM 510 software by visually identifying the top and bottom of cells using actin and nucleus staining as references based on their membrane proximity.

**Mounting.** Mowiol solution was prepared by dissolving 20 g of polyvinyl alcohol (Sigma, St. Louis, MO) in 80 ml mHBSS by stirring for about 1 hr on a heating plate, after which 40 ml glycerol was added and stirred for another hour at room temperature. Finally, 0.9 g of N-propyl-gallate was added as an anti-fade agent to the solution and stirred while protected from light on a heating plate until dissolved. Mowiol mounting medium was stored at 4°C in a brown amber bottle protected from light. Coverslips were mounted cell-side up on a Superfrost slide between four dried nail polish drops placed outside the area of the cover slip to support a second coverslip to be placed on top of the cell-seeded coverslip, thereby preventing cell distortion in the z axis due to the coverslip weight. A drop of Mowiol (refractive index 1.38 close to that of water) was placed on top of the cells and the second coverslip, a larger square 22 mm wide no. 1.5 coverslip (Corning, Actin, MA) was placed on top. This coverslip was small enough (22 mm wide) to limit possible deformation due to the lens objective. Once dried, the system was sealed with clear nail polish.

**Directed Cell Migration Studies in 3D**

**Chemotaxis.** BMSCs chemotaxis was performed in Costar Transwell (Corning, Acton, MA) systems with polycarbonate membranes bearing 8 μm diameter pores. Human PDGF-bb (R&D Systems, Minneapolis, MN), known to be involved in wound healing and chemotaxis [Lepisto et al., 1992], was used as chemoattractant for rabbit BMSCs at concentrations of 100, 50, 10, 1, 0.1 PDGF-bb ng/ml. Cell densities of 300,000, 150,000, 60,000, and 30,000 cells/well were used (data not shown) to identify an optimal density of 50,000 cells based on maximal chemotactic index and ease of counting without excessive cell overlap. Chemokinesis was evaluated by adding to the upper well the same concentration of PDGF-bb as in the lower wells. Cells were placed in the upper well of migration inserts 30 min before adding chemoattractant in the lower well, to allow cells to settle prior to forming the gradient. At 30 min,
since the bottom wells of this system are accessible without removing the cell-containing inserts, media was removed and 500 μl aliquots of chemotactic agents were gently added. Negative controls consisted of filling the basal wells with the same media in which cells are seeded in the apical wells, DMEM low glucose with PS without FBS.

Processing and Mounting of Membrane Inserts. Inserts were fixed at varying time points after gradient formation during migration. Samples were processed for quadruple labeling at 30 min, 1, 2, 3, and 4 hr. Membranes were processed as described above for coverslips, by adding reagents in the apical and basal chambers of the system to ensure homogenous labeling. Wells were filled and inserts containing membranes were passed sequentially from one well to the other. Once stained, membranes from the inserts were cut from their plastic support with a scalpel blade. Membranes were gently placed on a slide with the lower surface containing migrated cells facing up, between four dried nail polish spacers as described above. One drop of Mowiol was added and the system was then covered with a no. 1.5 square 22 mm wide coverslip.

Confocal Microscopy and Image Processing. Fluorescence imaging was carried out using an LSM 510 META Axioplan 2 confocal laser scanning microscope (CLSM) with a C-Apochromat 40X/1.2 water immersion objective (Carl Zeiss, Germany). Meta analysis of the four fluorochromes were performed to select optical setup and minimize overlap as described previously [Blanc et al., 2005]. Probe characteristics and optical settings for the image acquisition are summarized in Table II. Photobleaching induced by four laser z optical sectioning (stack) of the samples was assessed by comparing labeling intensity from the first section to the last section of the stack and was found to be minimal (data not shown).

RESULTS
Colony Formation Efficiency of BMSCs

Adherence purified rabbit BMSC morphology was fibroblastic with good adherence and colonies varying in size at 14 days post-isolation. Colony forming efficiency (estimated colony number per initial inoculum cell number) with initial inoculum of 10⁷ cells was estimated at 2 × 10⁻⁶.

Processing Method Evaluation Using Triple Stain

For the initial testing of a broad range of protocols only actin and vimentin were labeled along with the nucleus. Tubulin, being the more labile element of the cytoskeleton, is reportedly poorly preserved in alcohol or
PFA based methods of fixation [Baccalao et al., 1995] and was only labeled later using methods that were prescreened by triple labeling.

**Precipitating Agents.** Methods using precipitating agents such as ice cold methanol or acetone/methanol for permeabilization and fixation completely prevented actin labeling (Fig. 1A). These agents however preserved vimentin and provided a 2D rendition of the intermediate filament network. Cell height measurements indicated that these agents caused cell flattening that is not acceptable for 3D imaging (similar to Fig. 3C).

**Paraformaldehyde.** Effects of PFA on BMSC cytoskeleton preservation were analyzed for methods incorporating detergent permeabilization before, during or after fixation. Permeabilizing the cells before fixation in the delayed fixation method (no. 4 in Table I) caused cells to detach slightly from the substrate (Fig. 1B) revealing actin and vimentin networks with loose cytoplasmic extensions folding around each other and without clear stress fibers but rather a diffuse network of MF and IF. Vimentin also showed diffuse staining indicative of collapse or depolymerization due to this delayed fixation procedure. Permeabilization after fixation (no. 3 in Table I) flattened the cells in a manner similar to precipitating agents and signal intensity in this case was faint probably due to steric hindrance from cellular protein trapped inside cells by fixation prior to permeabilization (Fig. 1C). Instantaneous perm/fix (nos. 5 and 6 in Table I) yielded labeling that was bright and highlighted well preserved structures with high resolution and with minimal actin dots or loose extensions, for all samples, independent of concentrations of agents within the tested ranges (Fig. 2; top row). However, these methods also induced undesirable cell flattening (Fig. 3C) that was evident when examining stacks and finding little pattern differences in the z-direction, suggesting a flattened structure. Cell height measurements confirmed the cell flattening (Fig. 4) for both versions of the instantaneous PFA methods (nos. 5 and 6 in Table I).

**Glutaraldehyde.** GA fixation was either performed after permeabilization that contained a small amount of stabilizing fixative (smooth fix nos. 7-10 in Table I) or instantaneously with detergent permeabilization (nos. 11 and 12 in Table I). In the first case (smooth fixation) both actin and vimentin networks were complete and easily discernible for all sub-conditions of this GA-based smooth fixation (Fig. 2; second row), whether using 0.5 or 2% of either Octyl Poe or Triton X-100. Networks showed pattern variation with cell depth which was indicative of good cell preservation and cell heights were similar to live cells (Fig. 4). The instantaneous GA perm/fix method using both low detergent and low fixative concentrations simultaneously (nos. 11 and 12 in Table I), revealed a nicely preserved actin network, despite some punctate staining, while the vimentin network presented as a relatively contracted network, but with good labeling detail. The vimentin network seemed to be composed of finer fibrils than with other methods, i.e. with no preponderant filaments, but still radiating throughout the cytoplasm from the nucleus and lightly retracted without direct contact at cell edges and extensions (Fig. 2; third row). This disrupted appearance for vimentin was evident when using all 4 GA methods, whether smooth (nos. 7-10) or instantaneous (nos. 11 and 12) with either mMBS or Kpipes as buffer. Nonetheless, this gentle fixation led to the best preservation of cell volumes (Figs. 3B and 4). The addition of PFA to this fixation method (no. 13 in Table I) resulted in increased resolution and preservation of the vimentin network in addition to preservation of cell height (Figs. 2 (bottom row) and Fig. 4). Actin dots (arrows in Fig. 2) were seen in all samples with GA as fixative in either prefixation or instantaneous methods, as opposed to instantaneous fixation in PFA.

**Quadruple Cytoskeletal Labeling.** Emission spectral analysis (META detector) of each probe present alone in monostained cells excited by each of the four laser lines used in this study confirmed the absence of signal overlap between fluorophores under the optical setup used for imaging cells labeled for actin, vimentin, tubulin and nucleus as described previously [Blanc et al., 2005]. The use of 0.5 % GA 0.3 % PFA 0.3% Triton X-100 (no. 13; Table I) followed by labeling with the procedure described in the Methods provided simultaneous high resolution detailed networks of actin, vimentin and tubulin along a finely localized counterstained nucleus in both cells cultured on glass coverslips or in polycarbonate porous membranes (Fig. 5). MFs were bundled up in stress fibers, characteristic of spread cells. Intermediate filaments radiated throughout the cytoplasm from the perinuclear envelope while tubulin spread out as a well defined network originating from a more focal point near the nucleus usually identified as the MicroTubule Organizing Center (MTOC) or centrosome.

**Temporal Analysis of the Cytoskeleton in BMSCs Migrating Through a Porous Membrane**

BMSCs showed directed migration towards certain concentrations of hPDGF-bb with a maximum chemotactic index at a PDGF concentration of 50 ng/ml (Fig. 6). Chemokinesis controls showed no increase in migration at any concentration. This 50 ng/ml concentration was further used here in quadruple labeling of BMSCs migrating through porous membranes. The tem-
Fig. 1. Poor preservation and labeling of the cytoskeleton of BMSC in monolayer is obtained when processed with methods that are traditionally used in 2D light microscopy. Details of the processing methods are in Table I and the optical setup for this 3 color method (actin = green, vimentin = orange, nucleus = blue) is summarized in Table II. (A) Cold methanol fixation (no. 2; Table I) prevented actin labeling. (B) Delayed fixation (no. 4; Table I) by applying 4% paraformaldehyde after 0.3% Triton X100 permeabilization caused cells to detach from the substrate. (C) Standard fixation (no. 3; Table I) with 4% paraformaldehyde followed by 0.5% Triton X100 permeabilization yielded very faint labeling. Bar = 10 μm.

Fig. 2. Acceptable methods for 2D preservation and labeling of the cytoskeleton of BMSC in monolayer. Details of the processing methods are in Table I and the optical setup for this 3 color method (actin = green, vimentin = orange, nucleus = blue) is summarized in Table II. Actin and vimentin at one optical section are shown separately followed by these two images superposed with the nucleus. All four of these methods where detergent is mixed with fixative yielded good preservation, labeling and detail for these networks when viewed in two dimensions. Numbers in parenthesis refer to processing method in Table I. Arrows indicate actin dots. Bar = 10 μm.
poral progression of migration was observed by fixing membrane inserts in 0.5% GA/0.3% PFA/0.3% Triton X-100 at times of 30 min, 1, 2, 3, and 4 hours after addition of chemoattractant. The first time point 30 min after addition of PDGF, clearly indicated that directed migration was in progress, with small actin and tubulin cytoplasmic extensions already reaching the lower surface of the membrane (Fig. 7). Vimentin signal was also visible in the center of some pores, but only as a loose network, not attached to the substrate (Fig. 7). Cellular extensions appeared to line the pore walls adopting a cylindrical 3D or toroidal morphology. In corresponding magnified images of representative cells at 30 min, tubulin and actin filament were clearly defined at the leading edge of this toroidal structure of cells progression through the pore (Fig. 8), while the bulk of vimentin colocalized around the nucleus towards the initial side of the support. At this early stage, no clear attachment sites, identified by dense actin structure at the cell edge, are visible on the migrated side.

Subsequent time points from 1 to 4 hr after addition of chemoattractant show clear filamentous extension in 3D evolving actin stabilizing and anchoring structures on the migrated side of the membrane (1, 2 hr in Figs. 7 and 8). Tubulin colocalized with actin at earlier times, with a fine detailed network throughout the cytoplasm of cell extensions on the migrated side at all times. At 2 hr, cell spreading can be seen clearly where actin and tubulin extend on the migrated side. Vimentin networks are also visible, but are mostly still concentrated within the pore around nuclei. It should be noted that cell nuclei are visible on the migrated side in about 10% of cells at 2 hr, meaning that some nuclei pass more freely through the pores, with other cells pull on the nucleus remaining in the pore. At 3 hr, most cells adopt a typical BMSC monolayer non-confluent morphology, with star shaped extensions, with parallel actin stress fibers along those extensions, terminating in actin rich domains (Fig. 7).

Fig. 3. Cell height measurements for the assessment of 3D morphological preservation of processed BMSCs in monolayer. (A) X-Z view of an Imaris 3D reconstruction of live BMSC stained with membrane lipophylic dye DiI. Cell height was assessed by distance measurements aligned with the Z-axis between points (arrowheads) on X-Z and Y-Z ortho slices. (B,C) Orthogonal views showing central X-Y plane flanked on the right by Y-Z plane and below by X-Z plane of a 3D stack of fixed cells labeled for actin (green), vimentin (orange) and the nucleus (red). (B) A well preserved 3D morphology can be appreciated in cells treated with the instantaneous GA method (no. 12) where maintenance of nuclear height is observed in X-Z and Y-Z planes. (C) In contrast flattened nuclei and cells are seen after fixation using the instantaneous PFA method (no. 5), although the two dimensional X-Y projection can appear highly resolved. A similarly flattened morphology was also observed with precipitating agents such as acetone/methanol. Bar = 10 μm.

Fig. 4. Cell height measurements on live cells (DiI) and after various fixation methods. Cell heights were measured using X-Z and Y-Z vertical sections of randomly selected cells. Cell flattening was also visually evident by simple observation. *P < 0.05 compared to DiI on live cells using Student’s T-test. Numbers in parenthesis refer to processing method in Table I.
riphery of the pore in which the nucleus is still present (3 hr; Fig. 8). Actin networks in these cases radiate from the pore as does the tubulin network, with both networks still very much present inside the pores. At 4 hr, cells clearly exhibit monolayer type morphologies (Figs. 7 and 8) with extensive networks for all three elements extending in the cytoplasm similar to those of monolayer cells (Fig. 5). Cells also start to gain height and morphology similar to that in monolayer.

**DISCUSSION**

In this report we have imaged changes in the organization of the nucleus and cytoskeleton during 3D migration by developing a method that labels all three main cytoskeletal elements simultaneously with the nucleus of BMSC while assuring preservation of cell structural integrity. The advantages of this procedure lies in its ability to permit detailed structural observations of these elements in single cells, allowing observation of possible interactions between components in a three-dimensional environment more representative of in vivo migration versus two dimensional assays.

**Optimal Processing and Imaging Methodology**

After screening several methods we found that the instantaneous permeabilization-fixation approach using both glutaraldehyde (GA 0.5%) and paraformaldehyde (PFA 0.3%) with Triton X-100 (0.3%) for 20 min (no. 13 in Table I) achieved optimal cell structure preservation in 3D together with ideal labeling intensity and fine detail resolution when used in our quadruple labeling of...
Fig. 7. Time series of BMSC migration through a porous polycarbonate membrane in response to 50 ng/ml hPDGF in a transwell insert. Membranes were fixed at the indicated times and mounted upside-down to illustrate the temporal progression of migration along the Z axis to the lower surface. Inserts were processed using the instantaneous perm-fix with 0.5% Glut/0.3% PFA/0.3% Triton X100 and then labeled for actin (green), vimentin (yellow), tubulin (false color magenta) and the nucleus (red). The lower surface of the membrane was imaged by CLSM and pores (white as indicated by the arrow) were also imaged in nonconfocal transmission mode. After 30 min of migration, actin and tubulin are both seen on the migrated side to be leading cell progression through the filter. Vimentin is also visible in some pores, but only as a loose network in the center of the pores that is not attached to the pore wall. Migration progresses steadily and at 3 and 4 hr, cells have attained a typical monolayer morphology with spread bipolar morphology, actin stress fibers and a large and relatively central nucleus. Bar = 10 μm.
Fig. 8. Cytoskeletal organization and distribution in 3D for BMSCs migrating through porous polycarbonate membranes in response to 50 ng/ml hPDGF in a transwell insert. Membranes were fixed at the indicated times using the instantaneous perm/fix (no. 13 in Table I) with 0.5% Glut/0.3% Para/0.3% Triton X100 and then labeled for actin (green), vimentin (yellow), tubulin (false color magenta) and the nucleus (red) and mounted upside-down to illustrate the temporal progression of migration to the lower surface along the Z axis. Membrane pores were visualized in nonconfocal transmission mode (white). Orthogonal views are shown with the central X-Y plane flanked on the right by the Y-Z plane and below by the X-Z plane taken from 3D stacks of representative cells on the membrane. X-Y images are projections of superposed sections including that part of the cell that has emerged on the underside of the membrane, for a total thickness indicated by the small parallel white lines in the X-Z and Y-Z views in the lower right corner of each image. Yellow arrows indicate the direction of migration for both X-Z and Y-Z planes of the orthogonal view. Actin and tubulin filaments are the earliest to traverse the membrane, seen clearly at 30 min. Dense actin bundles are clearly visible at 1 and 2 hr from various perspectives (white arrows). At 2–3 hr, the cytoskeleton begins to radiate outwardly from the pores, and depict the cell pulling on its nucleus, which characteristically is the last to migrate through the pore. At 4 hr, most of the nuclei have traversed the pores. Bar = 10 μm.
cells during migration. Although in this case both Triton X-100 and Octyl poe showed similar results in the smooth GA methods, for which the use of Octyl Poe was originally recommended [Baschong et al., 1999], Triton X-100 only was used in the instantaneous fixation methods based on previous experimental data and reports showing Octyl Poe induced cell collapse in the z axis (not shown) as well as cell detachment and even cells lysis [Bacallao et al.,1995; Baschong et al.,1999]. As anticipated, traditional methods used for 2D microscopy such as PFA fixation before or after permeabilization and simple methanol or acetone-methanol perm-fixation, negatively affected 3D cell structure and overall network architecture, consistent with other studies using diverse cell types [Bacallao et al. 1995; Arcangeletti et al., 1997]. In our study, Triton X-100 was used instead of Octyl poe based on experimental data and reports showing Octyl Poe induced cell collapse in the z axis (not shown) as well as cell detachment and even cells lysis [Bacallao et al.,1995; Baschong et al.,1999]. Techniques using instantaneous and smooth perm/fix with either GA or PFA revealed actin and vimentin as two separate but interwoven networks. Generally, these images show cytoskeleton properties expected for bone marrow stromal cells and other fibroblast-like cells, such as MSC hallmark actin stress fibers [Kinner et al., 2002] and vimentin arranged in a perinuclear fashion with dense extensions throughout the cytoplasm [Baschong et al., 1999; Carnes et al., 1997]. Actin dots or punctate actin observed in some samples could indicate either incomplete extraction of monomers and oligomers prior to or during fixation [Steinmetz et al., 1997] or possible actin associated with adhesion complexes with the substrate [Lange
er et al., 2000]. As opposed to GA methods, PFA in these techniques however induced marked cell flattening. The use of both GA and PFA fixatives in the instantaneous perm-fix method (no. 13) prevented cell flattening while preserving structural details. In particular, vimentin was only well preserved when PFA was added to the instantaneous GA method. This instantaneous GA-PFA perm/fix method was chosen to examine additional staining of tubulin. The resulting quadruple labeling showed tubulin distribution as expected for fibroblastic-cells [Baschong et al., 1999]. The finely detailed images of this highly labile network confirmed the robustness of the instantaneous GA-PFA method by preserving network details, in both cells cultured on glass and on the polycarbonate membranes used for 3D migration studies.

**Directed Migration in 3D**

Our observations of cells migrating through porous membranes are compatible with some of the general macromolecular mechanisms seen in 2D directed migration, but with some important differences. As in 2D chemotaxis, the initial response of cells to chemoattractant is polarization and extension of protrusions in the direction of the incoming gradient [Firtel and Chung, 2000]. However, leading processes in 3D are not broad sheet-like lamellae as seen in 2D [DeMali and Burridge, 2003], but rather a three dimensional crawling cylindrical structure seen from below as a toroid. While tubular cell pseudopodia have been observed in 3D matrices, [Friedli and Brocker, 2000; Martins and Kolega, 2006] none were found to have a toroidal morphology with the presence of MT at the very distal end. In comparison with the literature, these protrusions are difficult to classify. Although resolution does not permit visualization of individual filaments, actin organization at the very front at 30 min is not consistent with lamellae, which exhibit dense dendritic-like actin networks. Theses protrusions are also not consistent with filipodia, described as finger-like membrane extensions, displaying longitudinal actin organization in the direction of migration [Welch and Mullins, 2002] and devoid of MT [Etienne-Manneville, 2004]. In our images of 3D migration, membrane extensions do not appear finger-like, but are rather continuous in the form of a hollow tube, and MT are clearly present.

Although MT have been shown to be present in the leading edge of motile cells, most studies reveal MT slightly lagging behind actin-driven leading edges, and never in filipodia [Etienne-Manneville, 2004]. This study clearly shows the MT in the leading structures, reaching the very tip of the leading edge protrusion. Our observations of MT in these leading structures could imply a fundamentally different cytoskeletal architecture in cells migrating in 3D, with leading processes arranged differently than in 2D, compatible with distinct differences in structure and composition of other cytoskeletal elements such as adhesion complexes in 3D compared to 2D [Cukierman et al., 2001]. The consistent presence of MTs at the leading edge and clear orientation toward the direction of migration also highlights their essential role in directed migration as hypothesized in early studies [Vasiliev et al., 1976]. Emerging direct evidence now shows that MTs influence cell polarization and directed cell motility by crosstalk and interaction with actin via modulation of common effectors [Nelson, 2003; Ridley et al., 2003; Etienne-Manneville, 2004; Watanabe et al. 2005].

The temporal and spatial colocalization of MT and MF at the leading edge in our study underscores this close association and further agrees with studies observing MT penetration into active lamellipodia, suggesting that MT polymerization could drive actin-based protrusion growth [Waterman-Storer and Salmon, 1999] and play a direct role in cell traction [Dujardin et al., 2003].

Our study permitted the observation of formation and maturation of contacts between cells and attachment sites during migration. Once protrusions reach the other
side of the membrane, we observed a dense actin network to start forming at the edge around the pore, resembling a lamellipodia, described earlier as flat broad extensions with a meshed actin network and actin-based attachment sites [Small et al., 1999b]. The formation of actin spots at 1 hr at the lamellipodia leading edge are also representative of actin-based adhesion complexes [Small et al. 1999a], and are in accordance with the time required for assembly of such structures in trypsinized fibroblasts [Dourdin et al., 2001]. Adhesions in the form of focal complexes at the leading edge are typical of slow migrating cells such as fibroblasts, whereas rapidly migrating cells display very small submicroscopic adherions [Ridley et al., 2003]. Current evidence suggests that these structures act as precursors for focal adhesions [Rottner et al., 1999], a more complex type of adhesion seen in crawling cells, defined as dense actin structures at the end of long cell extensions marking the termini of stress fibers [Firtel and Chung, 2000]. Observation of these latter structures at 2 hr suggests that such a transition occurs in this type of directed migration in 3D.

The restricted pores in this system could induce mechanical tension in the cytoskeleton by resisting nucleus translocation. Although deformable, the nucleus is less so than the cytoplasm [Yamauchi et al., 2005]. Contrary to 2D and 3D models [Friedl and Brocker, 2000] of moving fibroblasts, the nucleus in our images is not at all centrally located in cells during migration, but lags behind until the very end, indicating difficulty in translocation. Nucleokinesis, or nuclear translocation, is mostly studied in dividing yeasts or fungi [Morris, 2000; Szabo et al., 2004] and remains poorly elucidated. Most studies suggest MT interactions with MF and cortical motor proteins as key elements in nuclear positioning [Starr and Han, 2003; Xiang and Fischer, 2004]. In cell migration, however, few studies have examined the way in which the nucleus moves within the cell and none in 3D. Most data on mammalian cells come from work on neurons that suggest MT in the leading portions of neurons are directly involved in nuclear movement by centrosome pulling and interactions with nuclear associated motor-proteins [Morris et al., 1998; Shaar and McConnel, 2005]. The narrowness of the environment inside the membrane pore renders difficult the identification of the MTOC by β-tubulin labeling alone as in monolayer cells (Fig. 5), however observation of the centrosome would be possible by labeling it with markers such as γ-tubulin or pericentrin. This is a limitation of the current study but the extensive network formation of MT and MF in front of the nucleus in the z axis, and the ensuing nearly perfect radialorganization of these networks towards the nucleus (still in the pore at 3 hr post-migration), suggests a similar form of active nuclear transport, possibly involving MF and MT. The 3D method with a relatively small pore size is therefore well suited for the analysis of nuclear translocation during migration, as pores create an artificial resistance to free nuclear translocation, hence possibly amplifying the processes involved in its active transport in physiological situations.

IFs are also involved in cytoskeletal cross-talk as new evidence implicates them in processes such as assembly of adhesion complexes [Gonzales et al., 2001] and in directed migration [Eckes et al., 1998, 2000]. In contrast, an organized IF network was not observed in our study until cells had nearly completed migration across the membrane. This IF network formation is concordant with kinetic studies on trypsinized cells [Prahland et al., 1998; Helfand et al., 2004], where a non filamentous juxtanuclear vimentin cap formed after 30 min and full network formation only at 3–4 hr. Taken together, in a process involving physical forces, the actual complete migration of the cells in the absence of an organized network, similar to what could be seen by using a chemical disturbing agent, these data suggest that IF play no apparent role in this type of 3D migration. Our results are congruent with the 2D case as observed in the study using vimentin nul fibroblastic cells [Holwell et al., 1997].

The overall migration process through a 10 μm thick membrane points to another distinct feature and possible limitation of our study related to the geometry and short migration distance (10 μm) since cell translocation across the membrane occurs with only one cycle of the normally cyclical process that is to be coordinated seamlessly and continuously [Lauffenberger and Horwitz, 1996]. Stationary focal complexes at the leading edge of fibroblasts upon which the cell body and nucleus translocates and finally disassembles with the tail [Zamir and Geiger, 2001] were not observed in our system. Observations suggests rather that the initial focal complexes near the pores move away as cell edge progresses, extending radially from the pore, with no cell body translocation occurring over adhesions at any point. The general migration process in this type of 3D system could be divided into three overlapping phases, with the first step being the axial migration of the cell leading processes across the membrane. This would be followed by a radial migration on the under side of the membrane, similar to cell spreading, with detachment of adhesions site nearest to the pores and new formation at the edge further out on the membrane [Helfand et al., 2004]. Notably, filipodia can grow as long as 10 μm [Welch and Mullins, 2002] and since this corresponds to the membrane thickness, it is possible that such structures could extend fully from either side of the membrane, adhere and move the cell across by successive traction from the spreading front, promoting the last phase of cell rear and nucleus translocation across the pore.
CONCLUSIONS

Optimized methodology has been developed to observe all three cytoskeletal networks and the nucleus in a time series of 3D migration across a porous membrane. Novel observations point to several differences compared to 2D migration, namely a pivotal role of MT in the 3D process and previously unobserved lag of nuclear translocation. This method can be further applied to observe relationships with cytoskeleton associated proteins in terms of location and associations within the cell motile machinery. Our study provides a tool that, together with live cell studies, molecular genetics and biochemical approaches, will aid in the quest to achieve an integrated view of directed migration processes under physiological conditions.

REFERENCES


