Multiframe particle tracking in intravital imaging: defining Lagrangian coordinates in the microcirculation

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The cellular composition of the microcirculation creates blood flow that can be unsteady and nonuniform. To obtain information about nonuniform cellular trajectories, we describe in vivo imaging techniques that provide both detailed tracking of individual particles as well as an approach to simultaneous multicolor particle tracking. Particularly relevant to biologic systems, Lagrangian methods provide information about the fate of individual particles and flow in the system.

INTRODUCTION

Conventional Eulerian velocimetry techniques provide a detailed assessment of the velocity field at a particular point in space and moment in time. This is achieved by measuring the spatial displacement of a large number of particles (1) obtained in two separate images at a known time interval. In

many applications, these techniques can provide a detailed snapshot description of the flow field.

Blood flow, particularly in inflammation, can present a challenge for Eulerian approaches. Significant variability in local cell velocities has been observed in inflammation (2–5). Similarly, the cellular composition of the blood—including red cells and

leukocytes—can create blood flow that is nonuniform. The nonuniformity of inflammatory blood flow is illustrated by leukocyte trajectories that involve margination mural interactions and prolonged residence times.

To provide a description of unsteady and nonuniform flow fields, Lagrangian methods (6) track the movement of individual particles (7). An advantage of Lagrangian descriptions is that these methods provide more information about the fate of individual particles and flow in the system. The disadvantage of a Lagrangian approach has been the technical demands of tracking individual particles in rapid biologic processes. The description of Lagrangian coordinates in a biologic system such as the microcirculation requires (i) intensely labeled particles (or cells) to enable detection within tissues; (ii) a detection system with sufficient temporal and spatial resolution to track particles at velocities up to 5 mm/s; and (iii) image analysis software that permits the accurate plotting of Lagrangian coordinates from video streams composed of thousands of acquired images.

We have previously described nanoparticles developed for defining flow fields in the microcirculation (7). In this report, we describe the appli-

Figure 1. Simultaneously acquired image stacks acquired at two non-overlapping emission wavelengths. (A) Illustration of two simultaneously acquired image stacks. The images are acquired at alternating wavelengths (ex. 510 nm; em. 710 nm) and then separated into two image stacks that reflect the acquisition wavelengths. The analysis of the time-position maps in each stack demonstrates the paths of separate particles or cells—Cell no. 1 and Cell no. 2—obtained during the same interval in time. (B) Flow paths of separate time-position maps can be combined into a single map reflecting simultaneously acquired flow paths. Sequential images were obtained of green (em. 510 nm) and infrared (ex. 710 nm) nanoparticles in a flow chamber perfused at 500 μ L/s. The images at each wavelength were obtained as alternating images. Based on the detected wavelength, the processing algorithm pseudocolored the particles and plotted their flow path. The separately analyzed flow paths were overlaid to provide more detailed comparison of the simultaneously acquired Lagrangian coordinates. In this example, Cell no. 1 reflects the faster centerline flow velocity, whereas Cell no. 2 path reflects the slower marginal flow velocity.

cation of recently developed electron multiplying charge-coupled device (EMCCD) cameras and image analysis software for the definition of Lagrangian coordinates in the microcirculation. In addition to improved tracking of individual particles, these techniques permit the simultaneous tracking of multicolored particles *in vivo*. The ability to define Lagrangian coordinates of different types of blood particles will provide a better understanding of the dynamics of both plasma and cell motion in the microcirculation.

MATERIALS AND METHODS

Mice

Male Balb/c mice (Jackson Laboratory, Bar Harbor, ME, USA), with care consistent with guidelines of the American Association for Accreditation of Laboratory Animal Care (Bethesda, MD, USA), were used in all experiments.

Nanoparticles

The nanoparticles were developed by Molecular Probes (Invitrogen, Eugene, OR, USA) for intravascular particle tracking. These particles were of similar composition to those reported previously (8), but manufactured with superior fluorescent characteristics, smaller size, and low surface charge content (7). The surface charge of the nanoparticles used in this study ranged from 1.5 to 6.5 $\mu\text{Eq/g}$. The nanoparticles were labeled with green (ex. 490 nm; em. 520 nm), orange (ex. 545 nm; em. 570 nm), and infrared (ex. 675 nm; em. 700 nm) fluorochromes. Although particles from 40 nm to 2 μm were investigated, 500 nm particles were used in most studies.

EMCCD Camera

Videomicroscopy recorded 14-bit fluorescent images using a C9100-02 EMCCD camera (Hamamatsu Photonics, Hamamatsu, Japan). The C9100-02 has an air-cooled head and on-chip electron gain multiplication (2000 $\times$). Images with 1000 $\times$

Figure 2. Nanoparticles in the mouse colon microcirculation. (A) Green fluorescent nanoparticles (500 nm) in the colon mucosal plexus capillaries as well as in the larger submucosal artery and vein. A sample of the video stream can be viewed as Supplementary Movie S1, available online at www.BioTechniques.com. Note that the larger vessels appear dark because of the hemoglobin light absorption, but the structure of the smaller vessels is not discernable (scale bar, 200 μm). (B) Instantaneous velocity measurements of nanoparticles reflecting spatial variation in centerline and marginal particle velocities in an 80 μm colon vessel. The zero point is the axial centerline of the vessel, with particle velocities plotted as the apparent distance from the centerline. The data are fitted to a polynomial curve for comparison ($R^2 = 0.65$). (C–E) Images of 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled blood and infrared nanoparticles using multidimensional image acquisition of the colon microcirculation. CFSE is used to illuminate the structure of the small vessels and the topography of the microcirculation. In the video stream, the CFSE-labeled blood was imaged at a 1:3 ratio to enhance the tracking accuracy of the infrared nanoparticles (scale bar, 100 μm ; time-stamp, ms): (C) combined CFSE and infrared nanoparticles, (D) CFSE, and (E) infrared nanoparticles. Background was subtracted for presentation purposes.

1000 pixel resolution were routinely obtained at 50 frames per second (fps). Frame rates exceeding 100 fps were obtained with binning and subarrays. The images were typically recorded in image stacks comprising 30-s to 10-min video sequences.

In Vitro Flow Chamber

The technical specifications of the flow chamber has been previously described (9,10). Briefly, the design features included 0.5 mm holes in both inlet and outlet manifolds to rapidly stabilize laminar flow and permit the use of standard microscope slides. Rounded fluid capacitors positioned at the ends of the flow deck dampened eddy currents at the higher flows. The flow chamber was perfused with a NE-1000 withdrawal syringe pump (New Era, Farmingdale, NY, USA). In most

experiments, the perfusate was normal saline containing nanoparticles.

Intravital Fluorescence Labeling

A 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Invitrogen) labeling solution was prepared in dimethyl sulfoxide (DMSO) as described (11). The freshly prepared CFSE (400 μL) was injected into the tail vein of an anesthetized mouse over 2–3 min. The CFSE tracer (ex. 480 nm; em. 520 nm) was imaged with 25 nm band pass filters (Chroma Technology, Rockingham, VT, USA).

Intravital Microscopy System

The exteriorized colon was imaged using a Nikon Eclipse TE2000 inverted epifluorescence microscope using

Nikon Fluor water dipping objectives (Nikon, Tokyo, Japan). The intravital microscopy was performed by using a custom designed water immersion stage. The tissue contact area consisted of a vacuum gallery that provided tissue apposition to the objective without compression of the tissue. An X-Cite™ (Exfo, Vanier, QC, Canada) 120 W metal halide light source and a liquid light guide was used to illuminate the tissue samples. Excitation and emission filters (Chroma Technology) in separate specially adapted LEP motorized filter wheels were controlled by a MAC5000 controller (Ludl, Hawthorne, NY, USA) and MetaMorph® software 7.0 (Molecular Devices, Downingtown, PA, USA).

Multiframe Particle Tracking

Particle tracking was performed on distance calibrated and digitally recorded multi-image stacks. The image stacks produced a sequential time history of velocity and direction as the acquired images were time-stamped based on the 100 MHz system bus clock of the Xeon processor (Intel, Santa Clara, CA, USA). The multidimensional image acquisition application of MetaMorph permitted the use of different acquisition parameters; particularly, the acquisition of multiple wavelengths at variable ratios. The movement of individual particles was tracked using the MetaMorph object tracking applications. The intensity centroids of the particles were identified, and their displacements tracked through planes in the source image stack. For displacement reference, the algorithm used the location of the particle at its first position in the track. Each particle was imaged as a high contrast fluorescent disk. The image of the particle was tracked using a cross-correlation centroid-finding algorithm to determine the best match of the particle/cell position in successive images. With routine distance calibration, the overlay of the image stack provided a quantitative assessment of the particle/cell path.

Three-Plane Visualization

The image planes were analyzed by MetaMorph to provide three-

dimensional information. The View Orthogonal Planes command allowed examination of cross-sectional slices through the image volume in the XY, XZ, and YZ planes. The command permitted definition of X, Y, and Z coordinates of the active pixel location as it was displayed in each image window. The command was used simultaneously with the Measure XYZ Distance command to draw wireframe tracking lines that followed cells or particles through multiple planes of the stack. The cell/particle paths were viewed as an overlay on both the original stack and on the orthogonal plane stacks. XYZ coordinates were routinely exported for detailed analysis and flow modeling.

RESULTS AND DISCUSSION

The time resolution, or sampling (frame) rate, determines the ability of a video microscopy system to track the movement of a living specimen or rapid kinetic process. In studies of the blood circulation, the development of EMCCD cameras with sampling rates greater than 100 fps have enabled particle tracking at velocities compatible with the integral scale of the microcirculation (12). The reason for this improvement is that EMCCD cameras have significantly changed the traditional trade-off between light sensitivity and sampling rate. In the past, any increase in the frame rate would significantly increase the read noise of the camera. With on-chip multiplication, however, the signal detected by the camera can be boosted above read noise at any readout speed. By combining the electron multiplication feature with larger detection arrays and improved low light sensitivity, it is now possible to acquire Lagrangian coordinates in vivo.

Particle tracking requires a concentration of intravascular particles that optimizes sampling of the flow field, but minimizes overlapping particle images in the video stream. The technical improvements in camera speed and light sensitivities (e.g., frame rates exceeding 60 fps) have enabled the routine acquisition of simultaneous video streams of two different

fluorescent particles. The use of multi-color detection improves tracking fidelity as well as spatial sampling of the flow field. In addition, the separate tracking of two different labels offers the opportunity to track particles with different properties within the same flow field. For example, the movements of nanoparticles and blood cells should provide an informative comparison—particularly in conditions associated with leukocyte trajectories involving margination mural interactions and prolonged residence times.

Simultaneous tracking is the result of streaming video images being alternately acquired at non-overlapping emission wavelengths. Our optical system detects two distinct labels with excitation or emission filter changes at intervals that are only limited by the intensity of particle fluorescence and mechanical latency of the filter exchange system. After acquisition, the images are separated into two separate image stacks (Figure 1A). The distance-calibrated and time-stamped image stacks produce time-position maps of two distinct particles (cells) at the same region in space and during the same interval of time (Figure 1B).

Particle (cell) tracking in vivo may reveal local variation in flow velocity and cell movement; however, the time-position maps can be difficult to interpret without knowledge of the microvessel conduit and the flow field (Figure 2, A and B). To define the structure of the microcirculation as well as provide a measure of the overall velocity field, we intravenously inject the fluorochrome CFSE (ex. 480 nm; em. 520 nm). CFSE labels red and white blood cells as well as endothelium, providing an overview of the structure of the microcirculation (11). Because the CFSE-labeled particles produce overlapping images that limit the accurate assessment of particle tracking, we co-inject infrared (ex. 675 nm; em. 700 nm) nanoparticles. The CFSE and the infrared nanoparticles can be independently recorded and tracked without overlap in their emission spectra. The streaming images acquired at a 1:1 or variable ratio can provide information on both individual particle behavior as well as system dynamics (Figure 2, C–E).

Regardless of the particle or cell being tracked, computer software is critical for both image acquisition and image processing. Scientific imaging software, such as MetaMorph, not only controls the filtering and timing of image acquisition, but also annotates the image with the acquisition parameters. After acquisition, centroid-finding tracking algorithms applied to appropriately acquired images can convert gigabytes of image information into tables of numeric data that can be readily processed and analyzed. Thus, the acquisition time, emission wavelength of each image, and the distance calibration allow for the automated calculation of Lagrangian coordinates.

After acquisition of a video stream, the images are collated into image stacks. Each image within the stack defines particle (cell) coordinates in an XY plane (Figure 3A). Because the stack of images is acquired over time, it is possible to analyze the image stack in two additional planes. The YZ (or Y-Time) plane provides a cross-sectional view of the vessel at a particular point in space. The Y-Time plane provides information about movements that deviate from the axial flow stream. In contrast, the XZ (or X-Time) plane provides a longitudinal view over a given length of the vessel. The longi-

tudinal displacement of the particle (cell) as a function of time produces a measure of flow velocity (Figure 3, B and C).

Finally, the streaming acquisition of video images is potentially limited by image storage. Faster image acquisition has been matched with high-capacity graphics memory and high-speed hard drives. Using an appropriately configured imaging workstation, 5–10 min of streaming video can produce 30–60 GB of image data. The raw image data are typically analyzed and the Lagrangian coordinates calculated prior to archiving the video, because conversion of the video files to a lossy digital compression format such as ISO/IEC-14496 (MPEG-4) results in a degradation of the primary data. MPEG-4 format is an object-based compression in which individual objects are tracked separately and compressed together. Although the compression ratio (size of the original file to size of the compressed file) depends upon bit rate, the typical experiment can be reduced to file sizes that can be written to single- or double-density DVD disks for archiving.

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COMPETING INTERESTS STATEMENT

Y.-Z.Z. is employed by Molecular Probes (Invitrogen), the supplier of certain of the reagents used in this study. The other authors declare no competing interests.

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Figure 3. Three-dimensional visualization of the image stack. (A) The XY plane is the traditional image plane. The YZ (or Y-Time) plane provides a cross-sectional view of the vessel at a selectable point in space. The XZ (or X-Time) plane provides a longitudinal view over a given length of the vessel. (B) Orthogonal views of nanoparticles in the mouse colon microcirculation. Cells or particles can be tracked through multiple planes of the stack permitting a visual correlation in each plane. B1, XY image plane arithmetically combined over 250 images. The white objects represent stationary particles; B2, YZ (or Y-Time) plane of the axis of the artery. The slope of the diagonal lines represents the velocity of particles in the axial flow stream; B3, XZ (or X-Time) orthogonal plane of the same artery during the same time interval. Stationary particles in this cross-sectional view are observed as vertical lines as seen on the left margin of the image. (C) Scattergram of particle velocities in the centerline flow stream as a function of time, Z.

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