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Title
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Permalink
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ISBN
9781557529541

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Publication Date
2015

DOI
10.1364/ntm.2015.nm4c.1

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3D Nanoimaging and Detection of Molecular Flow using the nSPIRO Method

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Abstract: Detecting proteins dynamics within cells grown in 3D micro-environments is challenging. We developed a 3D nano-imaging technique to uniquely probes proteins in cells grown on collagen. Results show paxillin and actin diffusion rates are unique.

OCIS codes: 170.0170 Medical optics and biotechnology; 180.0180 Microscopy; 170.2520 Fluorescence Microscopy

1. Introduction

Cells sense their microenvironment by using a rich range of sensory mechanisms that allows them to change behavior, morphology and fate. It is well known that cells can reorganize their cytoskeletal structure depending on the surrounding extra cellular matrix (ECM) depending on the composition, topology, anisotropy, rigidity and pliability [1-5]. Limited imaging technologies capable of measuring protein interactions in real time and space for cells grown in 3D is a major impediment in understanding how proteins function under different environmental cues. To achieve this goal we have developed a nano-imaging technique capable of detecting molecular flows based on the orbital tracking method that can produce orbits modulated into a specific pattern. We have modified the original nano-scale precise imaging by rapid beam oscillation (nSPIRO) method to guide the laser beam orbit into a four leaf patterned shape [6, 7]. By multiplexing the pair correlation fluorescence (pCF) and the raster image correlation spectroscopy (RICS) methods with the 3D scanning, we can obtain information regarding protein binding, diffusion and aggregation in real-time and space.

Studying focal adhesion assembly and disassembly in 3D space will allow the understanding of tumor cell invasion in a more complex relevant environment. In this report we investigate paxillin and actin dynamics inside cells grown in the 3D microenviroment. Using orbital scanning FCS we found higher percentage of slow diffusing proteins at focal spots, suggesting assembling/disassembling processes. In addition, the RICS analysis shows paxillin aggregated predominantly at these focal contacts which are next to collagen fibers. At those sites, actin showed slower apparent diffusion rate, which indicated that actin is either polymerizing or binding to the scaffolds in these sites. Our findings demonstrate that by multiplexing these techniques we have the ability to spatially and temporally quantify focal adhesion assembly and disassembly in 3D space and allow the understanding tumor cell invasion in a more complex relevant environment.

2. Results

The nSPIRO method was set up on a commercial confocal, the Olympus FV1000 (Fig. 1). The internal galvano scanning mirrors were directly driven by an IOtech card (Measurement Computing, Norton, MA) with the pattern shaped algorithm to drive the laser beam into a modulated, circular or four-leaf pattern. Two-photon (880nm) or one-photon lasers (488nm) were coupled into the microscope either with a fiber or directly with optical mirrors mounted on an air table. The eGFP emission was collected using a 40x air 0.8 NA objective and with a bandpass filter between 505-605nm. To move the scanner in the z-axis, a piezo z-scanner (Phisik Instrumente, Auburn, MA) were driven by the IOtech axis card with nano-meter precision. Data were acquired and processed by the SIMFCS software (www.lfd.uci.edu, UCI, Irvine).

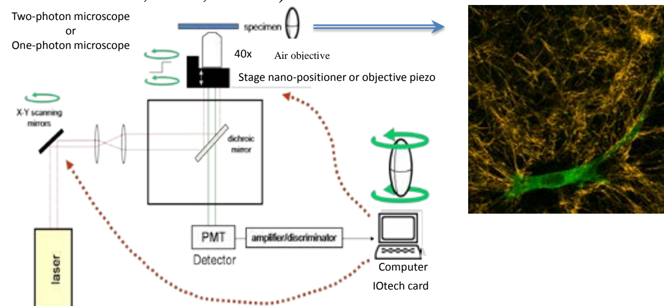

Figure 1. Microscope setup and image of a cell grown in 3D collage matrix. One-photon or two-photon excitation was used to excite actin-eGFP (488nm) and collagen (900nm).

Fig. 2 shows the raster scanned four leaf pattern of a cell protrusion with 128 pixels along the orbit. Four four-leafed orbits are scanned sequentially with a period of 8.2ms with distance or 0.5µm. From temporal cross-correlation of a pair of points at a given distance from each other, the pair-correlation function (pCF) analysis can define the diffusive route taken by molecules over many pixels (microns) along the line measured. When the distance between the two points is small, points are in within the PSF(Point Spread Function). This produces a correlation of the intensity fluctuations at very short time which is just the autocorrelation function (Fig. 2D, bottom). When the molecules diffuse further in space and time, the pCF will give rise to positive amplitude with a given time delay (Fig.2E). If there is a positive molecular flow, the amplitude will lower and the width of the Gaussian curve will narrow revealing fast fluid motion. Direction of the flow will be determined by calculating the pCF in the forward or backward directions (Fig. 2F).

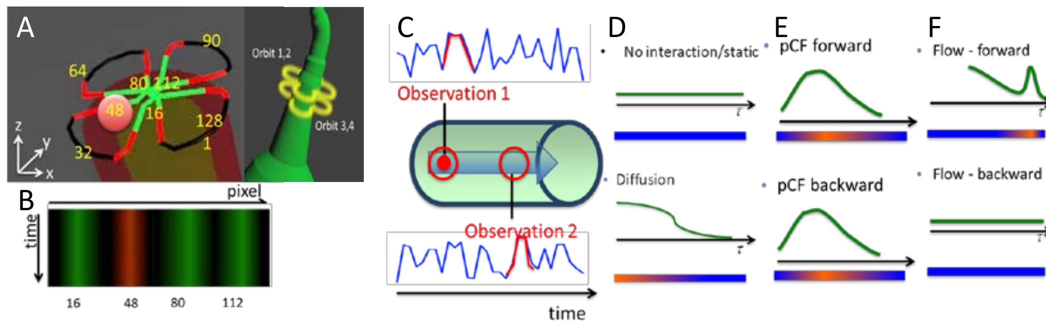

Figure 2. Patterned orbital scan and depiction of the pCF analysis. A) The circular scan is done with a four leafed shape. In position 48 along the scanned line a red object is detected. B) The fluorescence intensity along the line as a function of time is plotted on an intensity carpet and the red fluorescence is observed in position 48. D-F) Depiction of the pairwise correlation amplitude for each possible condition: no molecular motion or autocorrelation (D), pCF forward or backward normal diffusion with a given time delay (E), and forward or backward flow with a positive amplitude for direction of flow.

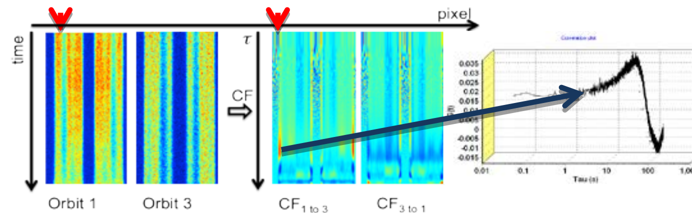

Figure 3. Actin Flow Measurement in 3D. pCF analysis was applied to orbits 1 and 3 of the four-leaf pattern. The pixel dwell time was set to 128 us with an orbit radius of 1 um. The Actin flow rate was calculated to be equaled to 1.5 µm/ min.

Figure 3 captures the positive molecular flow of actin molecules. Two orbits were used to calculate the actin flow rate of the distal tip of the cell protrusion.

Figure 4 shows the RICS analysis from the nSPIRO data set. In this case the orbit is scanned along one position on the cell protrusion. If the molecules diffuse fast, the correlation will be positive among adjacent lines along the x-axis. The correlation will thus shorten long the y-axis. If the diffusion rate is slow, the correlation will dominate along the y-axis.

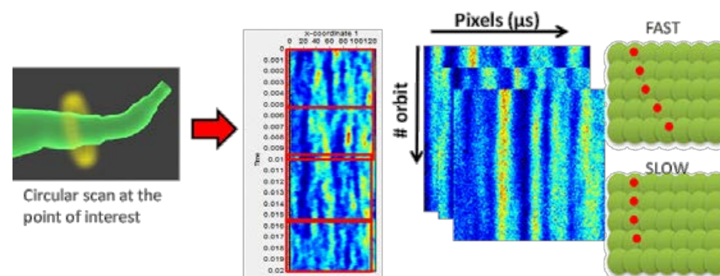

Figure 4. RICS along a line from the nSPIRO method. The orbit in this example is not smooth and only along one position along the cell protrusion. The intensity is plotted as a function of time and the time segments are cut into short time intervals or frames. The fluorescence intensities are correlated along the x and y axis as a function of time and space.

Table 1 shows the data obtained with the RICS along the nSPIRO line. Actin diffuses much slower than paxillin. Bound actin diffuses at a much slower rate of 0.21µm^2/s. Focal adhesions are much more difficult to detect in 3D. In

order to select region of positive focal adhesion contact with the ECM, the nSPIRO method was used to image at the nano-meter scale the position of the FAs. In table 1 there were not FA detected. Only fast diffusing paxillin was detected. However, actin will still polymerize to form the stress fibers required for cell motion. Together this data indicate that FA exsist but assemble and disassemble a much faster time than seen on 2D cell cultures

Protein	***Moving Average (S)***	***G(0)₁***	***D₁ (μm²/s)***	***G(0)₂***	***D₂ (μm²/s)***
Actin-eGFP	10	0.00084	13 ±1.2	0.0002	0.21 ±0.02
	50	0.000024	13 ±2.7	0.0002	0.12 ±0.06
Paxillin-mcherry	10	0.0004	17 ±5.3		NA

Table1. Diffusion rates of paxillin and actin labeled eGFP. Cells on 3D collagen were selected and nSPIRO was performed on a selected spot along the cell. For each data set a high pass filter subtracting out the immobile fraction and slow moving features of the cells were subtracted with either a 10s or 50s time scale. A total of 5 cells were measured for each experiment.

3. Equations

The pixel coordinate of the orbit is determined by Eq. 1 and 2. The radius r is the acquisition radius, and θ spans from 0 to 360°. Given that radius alternates four times during an orbital period the resulting shape is a four-leaf clover.

$$x=(r+r * \sin(4\theta))*\cos(\theta) \quad (1)$$

$$z=(r+r * \sin(4\theta))* \sin(\theta) \quad (2)$$

To calculate the pCF of molecular flows long two adjacent lines of the four-leaf clover shaped orbit we used Eq. 3 where Fa and Fb indicate the intensity in the upper and lower orbits, respectively. x is the pixel position, and τ is the correlation time:

$$G_{ab}(x,\tau) = \frac{< F_a(x,t)F_b(x,t+\tau) >}{< F_a(x,t) > F_b(x,\mathrm{t}) >} - 1 \quad (3)$$

Given that each pixel along the nSPIRO line has intensity fluctuation information, rows and columns along the intensity carpet can be correlated as a function or time and space. Each column (i axis) is the intensity along the circular orbit, and each row (j axis) represents an orbit taken at different time points. The spatial correlation function is given by Eq. 4 where ξ and ψ are the spatial increments in the i and j directions, respectively, and the angle bracket indicates average over all the spatial locations in both i and j directions.

$$G_s(\xi,\psi) = \frac{< I(i,j)I(i+\xi, y+\psi) >_{i,j}}{< I(i,j) >^2_{i,j}} - 1 \quad (4)$$

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