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Live cell imaging of paxillin in rolling neutrophils by dual-color quantitative dynamic footprinting (DqDF)

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Abstract

Objective—Neutrophil recruitment to sites of inflammation involves P-selectin dependent rolling. Quantitative dynamic footprinting is a useful tool to visualize the topography of the neutrophil footprint as it interacts with the substrate. However, elucidating the role of specific proteins in addition to topography requires simultaneous visualization of two fluorochromes.

Methods—To validate dual-color quantitative dynamic footprinting, mouse neutrophils were labeled with the membrane dyes DiO and DiI and perfused into microchannels coated with P-selectin-Fc. Footprints of rolling neutrophils were recorded as two separate images, one for each fluorochrome. To assess the localization of the cytoskeletal protein paxillin, we applied dual-color quantitative dynamic footprinting to DiO stained neutrophils of mice expressing an mCherry-paxillin fusion protein.

Results—The footprint topographies obtained from DiO and DiI in the plasma membrane were identical. The z-coordinates of the microvilli tips obtained with the two fluorochromes in the footprint were also identical. Paxillin was found to be localized to some, but not all ridges in the neutrophil footprint.

Conclusions—Our data suggest that the spectral properties of the fluorochrome do not affect the results. Dual-color quantitative dynamic footprinting will be useful for simultaneous visualization of two fluorochromes in the footprint of rolling cells.

Keywords

DqDF; P-selectin; footprint; TIRF

INTRODUCTION

The innate immune response following a bacterial infection or tissue injury involves neutrophil recruitment to sites of inflammation [29,51]. The recruitment process is a cascade-like process which is initiated by capture followed by P-selectin mediated rolling along the inflamed vascular endothelium [2,29]. P-selectin is stored in the Weibel-Palade bodies of ECs and translocated to the plasma membrane of cultured ECs at 20-50 molecules/ μm^2 following treatment with thrombin, histamine or other inflammatory mediators [6,23].

The excess membrane on the neutrophil surface is folded into interconnected ridges of structures known as ‘microvilli’, which are 200 nm long and 50-100 nm wide [17]. PSGL-1 is a ligand for P-selectin and is constitutively expressed on neutrophil microvilli [6,36]. P-selectin-mediated rolling is a result of rapid, yet balanced formation and dissociation of P-selectin-PSGL-1 bonds in the front and rear of the rolling neutrophil, respectively under the shear force and torque exerted by the flowing blood [14,22]. Over the last two decades, P-selectin mediated neutrophil rolling has been studied *in vivo* using intravital microscopy [8,12,55] and in *in vitro* flow chambers using trans-illumination [1,28,59], epifluorescence [50], and DIC microscopy [42,47]. These studies have provided cellular and molecular measurements that have led to the creation of *in silico* theoretical models [7,25,27,39-40] which provide invaluable insight into the molecular and cellular mechanisms that govern neutrophil rolling.

Recently, we developed qDF microscopy to study the footprints of EGFP-expressing or DiI-stained neutrophils rolling on P-selectin substrate at high shear stress [53]. qDF microscopy is an adaptation of TIRF microscopy, allowing real-time measurements of “height” of the hills and valleys in the footprint of rolling neutrophils. qDF revealed that a rolling neutrophil has a four-fold larger footprint than that predicted by theoretical models [39-40] as well as *in vivo* measurements [12,18]. In addition, qDF revealed 3-4 long tethers at the rear of each rolling neutrophil. It also provided the first experimental evidence that P-selectin-PSGL-1 bonds are relaxed in the front where they form, compressed in the center, and stretched at the rear of a rolling neutrophil where they eventually break away from the substrate [14,22,53].

Although qDF is a useful technique to elucidate biomechanics of neutrophil rolling, the current protocol is limited to visualizing only a single fluorochrome (EGFP or DiI) in the footprint of a rolling neutrophil. To further explore the cellular and molecular biomechanics of neutrophil rolling, we aimed to develop a technique to study the role of cytoskeletal and transmembrane proteins, which require visualization of two fluorochromes in the footprint, one for the topography and one for the molecule or organelle studied. Dual View is an optical coupler between the CCD camera and the microscope (Fig. 1) that allows the camera to simultaneously capture two perfectly registered images through two different fluorescence filters, so that each image shows one of the two fluorochromes. Dual View has been used previously for epifluorescence microscopy, for example, to study cellular division in fungi [38].

Paxillin is a scaffolding or adapter protein which is recruited to focal adhesions and mediates indirect interaction between the extracellular milieu and the cytoskeleton by facilitating the recruitment of several structural and signaling proteins to the focal adhesion [13]. Through interactions with these signaling and structural proteins, paxillin is known to coordinate the dynamics of cell spreading and migration by regulating the spatio-temporal activity of the Rho family of small GTPases [13]. Although paxillin is known to regulate adhesion, migration and spreading in fibroblasts [13,26,57] and lymphocytes [24,31-32,43], its role in neutrophils, which do not form focal adhesion complexes, is not clear. Some studies [10,16,19-21] have proposed a role for paxillin in β_2 integrin-mediated adhesion and spreading of neutrophils. This study was undertaken to develop and validate dual-color qDF (DqDF) and then to apply it to simultaneous real-time imaging of cell membrane topography and the distribution of paxillin near the membrane, as an example of biological experiments enabled by this technique.

MATERIALS AND METHODS

Reagents

Recombinant mouse P-selectin/Fc chimera was purchased from R&D Systems Inc. (Minneapolis, MN). Blocking mAb RB40.34 (rat IgG₁) against mouse P-selectin, and 4RA10 (rat IgG₁) against mouse PSGL-1 were purified from hybridoma supernatant at the biomolecular facility of the University of Virginia.

Mice

C57BL/6J mice (6-8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). Flag-mCherry-paxillin transgenic mice were developed at the School of Medicine, University of California San Diego. These mice were constructed using a transgenic vector comprised of a chicken actin promoter/CMV enhancer driving the expression of a loxP sequence-flanked GFP cDNA followed by a flag tagged-mCherry fused to the N-terminus of a mouse paxillin cDNA. Founder mice were identified by PCR of genomic DNA using primers to GFP sequence (sense: 5'GCACGACTTCTTCA AGTCCGCCATGCC3'; antisense: 5'CCGTTCTTCTGCTTGTCGGC CATGATA3') and visualization of GFP-fluorescence in various tissues. A founder line on C57BL/6J and Sv129/J mixed background was selected for ubiquitous GFP expression and Cre-dependent flag-mCherry-paxillin expression. This founder line was crossed with Tie2-Cre mice to create transgenic mice expressing flag-mCherry-paxillin. All mice were bred in a specific pathogen-free animal facility at the La Jolla Institute for Allergy and Immunology (LIAI). Mice were handled according to the guidelines set by the Department of Laboratory Animal Care at LIAI and all surgical procedures were done as per the guidelines in the protocol approved by the Animal Care Committee of LIAI.

Negative selection of bone marrow neutrophils

Neutrophils were isolated from the bone marrow of mice using immunomagnetic negative selection (EasySep mouse neutrophil enrichment kit; STEMCELL Technologies, Vancouver, BC). The femurs and tibiae of mice were purged with HBSS without Ca²⁺ and Mg²⁺ (GIBCO-Invitrogen, Carlsbad, CA) to collect the bone marrow cells. The cell pellet was collected by spinning the cell suspension at 300g (5 min, 22° C) in a centrifuge (Sorvall RT, Thermo Scientific, Asheville, NC). The cells were suspended in Robosep buffer + 5% rat serum (EasySep kit) at a concentration of 10⁸ cells/ml. The cell suspension was incubated with the negative selection Ab cocktail (EasySep kit) for 30 min at room temperature and then, washed in HBSS without Ca²⁺ and Mg²⁺ in the centrifuge at 300G (5 min, 22° C) and resuspended in roboset buffer + 5% rat serum at a concentration of 10⁸ cells/ml. The neutrophils were negatively isolated using ROBOSEP (STEMCELL Technologies, Vancouver, BC) and the EasySep kit. Negatively isolated neutrophils were washed with HBSS without Ca²⁺ and Mg²⁺ in a centrifuge at 300G (5 min, 22° C) and finally suspended in RPMI-1640 (GIBCO-Invitrogen, Carlsbad, CA) at a concentration of 10⁶ cells/ml and kept at room temperature.

Staining of neutrophils with DiI and DiO

Mouse neutrophils in RPMI-1640 at a concentration of 10⁶ cells/ml were incubated with membrane dye Vybrant DiI and Vybrant DiO from Molecular Probes Inc. (Eugene, OR) at a final concentration of 1μM for 20 min at room temperature in dark [53]. DiO and DiI are lipophilic dyes that uniformly incorporate into the cellular plasma membrane and have absorption and emission peaks, respectively, of 484 and 501 nm for DiO and 549 and 565 nm for DiI. The cells were washed twice with RPMI-1640 in a centrifuge at 300g (5 min,

22° C) and finally suspended in RPMI-1640 + 10% mouse plasma at a concentration of 2.5×10^6 cells/ml and stored at room temperature in the dark.

Microfluidic perfusion assay

A schematic diagram of the experimental set up is shown in Fig. 2. Coating of the cover slips with P-selectin and the assembly of the microfluidic device has been described previously [53]. A P-selectin coating concentration of 2 $\mu\text{g/ml}$ results in a site density of 20 molecules/ μm^2 [53]. The assembled microfluidic device had nine identical perfusion chambers with the P-selectin coated glass cover slip forming the bottom surface of each chamber (Fig. 2B). The inlet of the device was connected by tubing to a reservoir containing cell suspension (2.5×10^6 cells/ml in RPMI-1640 + 10% mouse plasma) while the outlet of the device was connected by a separate tubing to an outlet reservoir filled with PBS. The device has been calibrated previously to achieve physiological wall shear stress in the perfusion chambers as a function of the pressure difference across the inlet and outlet [53]. The desired physiological wall shear stress in the perfusion chambers was achieved by applying a well-controlled differential pressure between the inlet and outlet of the device as described before [53]. The pressure was generated hydrostatically by placing the level of liquid in the inlet reservoir above that in the outlet reservoir, using a vertical rail and a sliding stage (Fig. 2A). The microfluidic device was placed on the stage of the inverted TIRF microscope (see below) and the footprints of rolling neutrophils were studied using DqDF microscopy.

MAb treatment

The adhesion specificity was verified using mAb RB40.34 against mouse P-selectin and mAb 4RA10 against mouse PSGL-1. RB40.34 was mixed with the cell suspension and perfused into the microfluidic device at a final concentration of 30 $\mu\text{g/ml}$. RB40.34 abolished all rolling showing that the rolling was P-selectin specific.

Cells were incubated with 30 $\mu\text{g/ml}$ of 4RA10 for 10 min and then perfused into the microfluidic chamber. 4RA10 abolished all rolling showing that the rolling was mediated by PSGL-1 on neutrophils.

DqDF microscopy setup

DqDF setup was a modification of the single color qDF setup described previously [53]. The set up consisted of an IX71 inverted TIRF research microscope (Olympus America Inc., Center Valley, PA) placed on a vibration isolation stage (TMC, Peabody, MA). The neutrophil footprints were observed with a 100x NA = 1.45 Plan-Apochromatic oil immersion TIRFM objective. The microscope was equipped with a motorized stage and used 30 mW blue ($\lambda = 488\text{nm}$) and 20 mW yellow-green ($\lambda = 561\text{ nm}$) diode-pumped-solid-state lasers (CVI Melles Griot, Carlsbad, CA) as TIRF excitation light sources. The output of each laser was controlled separately. In DqDF experiments, the rolling neutrophils were simultaneously excited by the blue and yellow-green laser in the TIRF mode using two separate single-mode fibers entering the TIRF illuminator. The dual color images of the footprints of rolling neutrophils were captured using a DV2 (PHOTOMETRICS, Tucson, AZ) Dual View video coupler and a 16 bit digital CCD camera (HAMAMATSU C10600-10B ORCA- R^2) which was connected to a PC (Dell Precision T3400; Intel Core 2 Duo CPU-3.16 GHz; 3.25 GB RAM) through an IEEE1394b interface (see Fig. 1). A precisely registered dual color image has every pixel in the channel 1 exactly overlaid in the same pixel in channel 2 that requires perfect alignment of the DV2 with respect to the CCD camera. The DV2 consists of one aperture for the DV2 and the other for the CCD chip of the camera. The CCD aperture divides the CCD chip into two equal halves. The DV2 and CCD apertures were aligned with respect to one another first manually using adjustment screws

and then, digitally using the Dual View alignment toolbox available in SlideBook 5.0 software (Intelligent Imaging Innovations Inc., Denver, CO). As the emitted fluorescent light entered the Dual View, it was split into two separate light beams using a dichroic mirror, two reflecting mirrors, and two separate band-pass emission filters (ET630/75m, 592-668 nm; ET525/50m, 500-550 nm; Chroma Technology Corp., Rockingham, VT). The two beams were directed to two halves of the CCD chip (Fig. 1), thus creating two separate images (pseudo colored to look red and green in Fig. 1) on the CCD chip. After careful alignment of the DV2 with respect to the camera, the images captured by the two halves of the CCD chip were registered to each other with an error of less than a pixel. Each of the two images had a resolution of 672 (horizontal) $\times$ 1024 (vertical) pixels (1 pixel = 64 nm). Images were captured at a rate of 2 to 8 frames/s. The laser shutters and camera were controlled with the SlideBook 5.0 software with built-in features for image analysis. The absorption and emission peaks of the fluorochromes used in this study were, respectively, 484 and 501 nm for DiO, 549 and 565 nm for DiI, and 585 and 610 nm for mCherry. A TIRF incidence angle of $\theta = 70^\circ$ was used for both lasers in all DqDF experiments except for calibration as described previously [53].

RESULTS

Rolling of DiI and DiO stained neutrophils

Rolling of neutrophils labeled with both DiO and DiI was studied at a wall shear stress of 8 dyn/cm². DqDF images of the footprints of rolling neutrophils were captured at intervals of 100 ms as two separate and perfectly registered single-color qDF images corresponding to DiO and DiI, respectively at each time point (Fig. 3). For each time point, the two single-color qDF images were superimposed to generate a dual-color DqDF image. The dual-color DqDF and the two single-color qDF images at four consecutive time points of a neutrophil rolling on P-selectin (20 molecules/ μm^2) are shown in Fig. 3. The yellow color in the DqDF images (top row Fig. 3) indicates the regions in the footprint accessible to both DiO and DiI. The DiO and DiI are localized in the same cellular compartment (plasma membrane) and the yellow color is uniformly distributed (top row Fig. 3) indicating that the DiO and DiI are equally distributed in the plasma membrane and any internal membranes within 200 nm from the glass cover slip. This is further verified by the identical single-color DiO (middle row Fig. 3) and DiI (bottom row Fig. 3) qDF images of the same footprint at different time points.

Validation of DqDF

The DiO (middle row Fig. 3) and DiI (bottom row Fig. 3) qDF images at $t = 0.3$ s were processed to generate 3D reconstructions of the two footprints shown in Fig. 4A and B, respectively. The image processing was performed using custom designed codes in MATLAB (The MathWorks Inc, Natick, MA) as described previously [53]. Briefly, a single color 2D qDF image was converted into a 2D distribution of intensities where each element of the distribution corresponds to a pixel in the qDF image. The intensity distribution was converted into a distribution of z -distances above the cover slip as described [53] and plotted as a 3D reconstruction in MATLAB. The two 3D reconstructions clearly show the x - and z -coordinates of individual microvillus tips. A microvillus was defined as any protrusion longer than 25 nm in z -direction. The conical protrusions at $x < -4 \mu\text{m}$ (Fig. 4) are anchorage points of long tethers. Neutrophils rolling at 8 dyn/cm² have been shown to form long tethers at the rear and the anchorage points of these long tethers can be seen in qDF micrographs [53]. The x -, y -, and z -coordinates of the microvilli tips were extracted from Fig. 4A and B and plotted as scatter plots shown in Fig. 5A and B, respectively. The z -position above the cover slip of the individual microvillus tip in the DiO footprint (Fig. 5A) was comparable to that in the DiI footprint (Fig. 5B). The mean difference in the z -position

of microvilli tips between DiO and DiI footprint (Fig. 5) was 9.7 nm which was less than 4% of the maximum z -position (250 nm in Fig. 4A).

Colocalization of DiO and DiI

To quantify the colocalization of DiO and DiI in the plasma membrane, we correlated the DiO and DiI fluorescence intensities for every pixel in a DqDF image. The DiI fluorescence intensities of all the pixels in the DqDF image at $t = 0$ s in Fig. 3 were plotted as a function of the respective DiO intensities (Fig. 6A). The correlation plot was fit to a straight line using linear regression [41] and the slope, y-intercept and related statistical parameters were estimated (DiO-DiI cell 1 in Table 1). The correlation plot in Fig. 6A shows data points arranged as a cloud centered on a line indicating complete colocalization of two fluorochromes [4]. The goodness of the linear correlation was estimated by calculating the Pearson's coefficient (PC in Table 1). The estimated PC of 0.98 for Fig. 6A is close to the value of 1.0 which corresponds to complete positive correlation. The statistical significance of the estimated PC was evaluated using Costes' randomization technique available in the JACoP toolbox of NIH-Image J as described previously [4,11]. Briefly, DiO images ($n = 1000$) were randomly generated using the original DiO image and each random image was compared with the original DiI image to estimate a PC. The distribution of PCs from the randomized images was compared to the PC of the original DiO-DiI image pair and a P -value was calculated. The P -value negatively correlates with the probability of obtaining a PC by chance and a value of 100% (DiO-DiI cell 1 in Table 1) was obtained for Fig. 6A. A similar analysis was also done for the DqDF image at $t = 0.3$ s in Fig. 3 which yielded identical results (DiO-DiI cell 2 in Table 1). The residuals of the linear fit for the cell 1 and 2 are shown in Fig. 6B and C, respectively. Based on the DiO fluorescence intensity, the residual data was distributed into three intervals separated by the blue dotted lines in Fig. 6B and C. The average of residuals in each interval was compared with the residual value of zero (red line in Fig. 6B and C) using the one sample t -test [45]. None of the averages were significantly different from zero showing that the correlation is linear. An object-based method described previously [3-4] was also used to further verify the colocalization of DiO and DiI in the plasma membrane. The structures close to or larger than the optical resolution (~ 230 nm for TIRFM 100x objective, NA 1.45) were analyzed separately by drawing a vector across the structure and then plotting the normalized intensity ($I/I_{\max}$) distribution for both DiO and DiI along the vector. DiO and DiI are colocalized in a structure provided the true overlap distance between DiO and DiI intensity distributions at mid-height is larger than the optical resolution (~ 230 nm). The true overlap distances for all structures were observed to be greater than 230 nm (representative example shown in Fig. 6D-E). DiO and DiI have an overlap in their emission and excitation spectra which leads to spectral bleed through during simultaneous excitation and emission of the two fluorochromes. However, this problem was circumvented by reducing the power of both excitation lasers, and at the same time decreasing the loading concentrations of DiO and DiI used for cell staining. With this strategy, the bleed through between the two channels was comparable to the background signal.

Distribution of mCherry-paxillin in neutrophil footprints

Simultaneous two-color illumination and imaging enables new applications of DqDF. Here, we use the DiO membrane staining and fluorescence in the green channel to reconstruct the surface topography of neutrophil membrane and fluorescence in the red channel to visualize the distribution of mCherry-paxillin fusion protein relative to membrane topography. DqDF images of neutrophil rolling on P-selectin (20 molecules/ μm^2) were captured at intervals of 100 ms as two separate and perfectly registered single-color qDF images showing the distribution of DiO (pseudo colored green image) in the membrane and mCherry-paxillin (pseudo colored red image) in the cytosol, respectively at each time point. Again at each

time point, the two single-color qDF images were superimposed to generate a dual-color DqDF image. Dual-color DqDF and the single-color DiO and mCherry qDF images at a single time point are shown in Fig. 7A. The regions in the footprint where DiO and mCherry-paxillin are colocalized appear yellow. As DiO incorporates homogeneously in the plasma membrane, the DiO qDF image in Fig. 7A was processed to generate a 3D reconstruction of the footprint (Fig. 7B). Again, the side view of the 3D reconstruction shows the x and z coordinates of microvilli in the footprint. The 3D footprint was rotated clockwise by 90° about the x -axis to generate the bottom view and the mCherry-paxillin qDF image (red image in Fig. 7A) was overlaid. The resulting image (Fig. 7C) shows the localization of mCherry-paxillin relative to the membrane topography of the neutrophil footprint, i.e. localization of mCherry-paxillin on hills (microvilli) vs. valleys (cell body). Fig. 7C shows that mCherry-paxillin appears to be more concentrated in larger interconnected ridges, than single isolated microvilli defined as the ones with no neighboring microvilli within $0.5\ \mu\text{m}$ in their vicinity. Pixel coordinates of the tips of microvilli (peaks of hills in Fig. 7C) both within the interconnected ridges and the ones isolated were extracted from the 3D-reconstructions of two rolling cells. Average mCherry intensity was calculated over a square region of 5×5 pixels around the pixel corresponding to the coordinates of microvillus tip. The rationale behind selecting a 5×5 pixel square is that a microvillus which is $100\ \text{nm}$ wide [17,39] would be completely enclosed inside a $320 \times 320\ \text{nm}^2$ region (1 pixel = $64\ \text{nm}$). The average m-Cherry intensities of microvilli from two cells were separated into two distributions corresponding to the single isolated microvilli or the ones within the interconnected ridges. The two distributions were plotted along with their medians (Fig. 8A) and compared using the U -test of Wilcoxon-Mann-Whitney ($P < 0.01$) confirming that mCherry-paxillin is more concentrated in the interconnected ridges than single isolated microvilli. This shows that DqDF is a useful technique to assess the localization of cytoskeletal proteins relative to the membrane topography in the footprints of rolling neutrophils. Similar to Fig. 6A-C, the mCherry intensity of every pixel in the DqDF images of two different cells (one shown in Fig. 7A and other not shown) was correlated to the DiO intensity of the same pixel. The correlation plots of both the cells were fitted to a straight line and the slope, y -intercept and the statistical parameters were estimated (DiO-mCherry cell 1 and 2 in Table 1). The PC was estimated for both the cells and its significance was confirmed using Costes' randomization technique (Table 1). The residuals of the linear fits for the two cells are shown in Fig. 8B and C, respectively. Again, the residuals were analyzed in three intervals (separated by blue dotted lines in Fig. 8B and C) and the average residual of each interval was compared to zero residual (red line in Fig. 8B and C) using one sample t -test. The mean of the interval with DiO intensity greater than 150 was significantly different from the zero value ($P < 10^{-8}$) showing a nonlinear correlation of DiO with mCherry.

DISCUSSION

Here, we introduce DqDF which is an advancement over single color qDF [53]. We show that DqDF allows simultaneous visualization of two fluorochromes with nearly perfect registration in or close to the plasma membrane in the footprints of rolling neutrophils. Single color qDF was shown to be a useful technique to study the biomechanics of neutrophil rolling [53]. However, the role of different cytosolic and cytoskeletal structures as well as proteins cannot be investigated using single color qDF.

These results show that DqDF allows simultaneous visualization of two spectrally distinguishable fluorochromes in the footprints of rolling neutrophils with high temporal resolution, while preserving both the quantitative and qualitative traits of the single color qDF [53]. Identical 3D-reconstructions (Fig. 4) and coordinates of the microvilli tips (Fig. 5) revealed by DiI and DiO which have different spectral properties but localize to the same

cellular compartment (plasma membrane) show that DqDF depends only on the cellular location of the fluorochromes and is independent of the spectral and quantum properties of the two fluorochromes. These observations also show that DqDF satisfies the theory of TIRF microscopy [52]. This is further validated by the analysis shown in Fig. 6 and Table 1, which shows a linear correlation and complete localization of DiO and DiI which are localized in the plasma membrane (same cellular compartment). In contrast, we found a nonlinear correlation for DiO and mCherry-paxillin that are localized in the plasma membrane and cytosol (different cellular compartments), respectively, with mCherry more concentrated in the interconnected ridges compared to single isolated microvilli.

One application of DqDF is to study the localization of cytoskeletal proteins relative to the membrane topography in the footprints of rolling neutrophils. In the past, scanning electron microscopy (SEM) has been used to study the localization of β_2 integrins, L-selectin [15,17], and PSGL-1 [36] relative to the membrane topography of neutrophils. Importantly, however, unlike SEM, DqDF does not require the cells to be fixed and stationary and it can be used with live rolling neutrophils at a temporal resolution of better than 100 ms.

Recently, TIRF microscopy has been used to study the translocation of Syk from the cytosol to the plasma membrane and NF κ B from the cytosol to the nucleus in B- and CHO cells, respectively [56]. DqDF can be extended to study the recruitment of different signaling and cytoskeletal proteins to the plasma membrane of rolling neutrophils. The proposed model can detect the cytosolic protein of interest as an mCherry-fusion protein and GFP expressed homogeneously in the cytosol or plasma membrane. Alternatively, a cell expressing an mCherry-fusion protein of interest in the cytosol can be stained with DiO that incorporates into the plasma membrane.

DqDF can also be used to study the localization of membrane rafts in the plasma membrane. PSGL-1 is a transmembrane glycoprotein on the neutrophil surface that mediates neutrophil rolling on P-selectin both *in vivo* [37] and *in vitro* [36]. PSGL-1 is known to be clustered in membrane rafts on the tips of microvilli and this mode of presentation is known to facilitate neutrophil rolling [34-35,44]. Membrane raft imaging can be achieved by having GFP expressed homogeneously in the cytosol and staining the membrane rafts with Alexa-568-labeled cholera toxin (CTx) B subunit that binds to GM1 glycosphingolipid in rafts [48,54]. The GFP channel could be used in reconstructing membrane topography and the Alexa-568 signal to visualize the membrane rafts localization relative to the membrane topography. Alternatively, the plasma membrane can be stained with DiI and the rafts can be tagged with FITC-labeled CTx B subunit. The DiI in the plasma membrane can be used to reconstruct membrane topography and FITC can be used to visualize membrane rafts.

Another potential application of DqDF is in the study of time resolved Ca^{2+} flux in rolling neutrophils. Recently, TIRF microscopy was used to study the transient Ca^{2+} flux in neutrophils during the transition from rolling to arrest on glass substrate coated with E-selectin and ICAM-1 [46]. DqDF can be used to study the local Ca^{2+} flux relative to the membrane topography in the footprint of rolling neutrophils. Neutrophils can be loaded with a calcium dye, for example, Fluo4 AM, which allows visualization of transient Ca^{2+} flux and stained with membrane dye DiI. The DiI signal will be used in reconstructing the membrane topography and the Fluo4 AM signal will allow visualization of Ca^{2+} flux.

TIRF microscopy has been used in studying the dynamics of the recruitment of cytoplasmic vesicles to the plasma membrane in HL60 cells transfected with EGFP-Rab27a [5]. DqDF can be used to study the selective recruitment of cytoplasmic vesicles to different regions of the neutrophil footprint. This can be achieved by staining the plasma membrane of the transfected cells with membrane dye DiI. DiI signal could be used to reconstruct the

membrane topography of the footprint and the EGFP signal will allow studying the time resolved recruitment of vesicles relative to membrane topography.

We conclude that DqDF is a substantial advancement over the single color qDF because it allows time-resolved visualization of membrane-proximal cellular events, such as clustering and localization of cytosolic and cytoskeletal proteins relative to the 3D membrane topography in the footprints of rolling neutrophils. For the technique to be effective, however, the spectral bleed through must be minimized by using a pair of fluorochromes with well separated absorption and emission spectra. Currently, DqDF is limited to the visualization of only two colors and is thus limited to visualization of a single cytosolic or cytoskeletal protein in addition to the membrane topography. The technique could be expanded to 3 or even 4 fluorochromes, if their spectra are sufficiently separated and the fluorescence bleed-through is minimal, and simultaneous real-time fluorescence imaging in as many as 4 colors using a single camera is possible with the Quad View QV™ video coupler by Photometrics, Inc. In addition, the availability of photo-switchable [9,30] and photo-activatable fluorescent proteins [33,49,58] which can be genetically fused to proteins may allow simultaneous visualization of even more than four fluorochromes in the near future.

Legend for the cover art

Localization of mCherry-paxillin fusion protein (red) in the microvilli ridges (grey hills) in the footprint of a rolling neutrophil. Hills correspond to microvilli and the valleys to the cell surface. Neutrophils from an mCherry-paxillin transgenic mouse stained with membrane dye DiO and suspended in RPMI-1640 + 10% mouse plasma were perfused through the microfluidic perfusion chamber at a wall shear stress of 8 dyn/cm². DqDF image of a neutrophil rolling on P-selectin (20 molecules/μm²) was captured as two separate and perfectly registered single-color qDF images showing the distribution of DiO in the membrane and mCherry-paxillin in the cytosol, respectively. The DiO qDF image was processed to generate a 3D reconstruction of the footprint showing microvilli ridges and valleys. The image shown is the bottom view (grey) of the 3D footprint with the mCherry-paxillin (red) qDF image overlaid.

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ABBREVIATIONS

qDF	quantitative Dynamic Footprinting
DqDF	Dual-color quantitative Dynamic Footprinting
TIRF	Total Internal Reflection Fluorescence
PSGL-1	P-selectin Glycoprotein Ligand-1
EC	Endothelial Cell
CCD	Charged Coupled Device
Syk	Spleen tyrosine kinase

CHO	Chinese Hamster Ovary
DiO	Diocadecyl Oxacarbocyanine
DiI	Diocadecyl Indocarbocyanine

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Fig 1.

Scheme for the DqDF. Blue ($\lambda = 488$ nm) and yellow-green ($\lambda = 561$ nm) lasers incident at the glass ($n_1 = 1.52$)-cell ($n_2 = 1.37$) interface at an angle $\theta = 70^\circ$ which is greater than the critical angle $\theta_c = 64.33^\circ$. The incident beams undergo total internal reflection and establish respective evanescent waves (yellow) on the cell side of the cover slip. The evanescent waves established by the blue and yellow-green laser beams excite DiO and DiI in the plasma membrane of the neutrophil to emit green and red fluorescence, respectively. The emitted light passes through the DualView coupler where it is split by a dichroic mirror into two separate beams of fluorescent light emitted by DiO and DiI, respectively that are further rectified by two band pass emission filters (ET630/75m and ET525/50m, respectively). The two beams are captured by the two halves of the CCD chip, respectively and displayed as green (DiO) and red (DiI) pseudo colored identical images on the computer screen. n and λ represent the refractive index and wavelength, respectively.

Fig. 2.

Microfluidic device and perfusion assay. **(A)** A PDMS chip with nine identical flow channels engraved on the bottom surface is sealed against a glass cover slip with a magnetic clamp. The grey region on the cover slip is coated with 2 $\mu\text{g}/\text{ml}$ of recombinant mouse P-selectin-Fc. Mouse neutrophils are suspended at 2.5×10^6 cells/ml in RPMI-1640 + 10% mouse plasma and the suspension is flown through the microfluidic perfusion chamber. The desired wall shear stress is achieved by hydrostatic generation of a differential pressure, ΔP , between the inlet and outlet by raising the inlet reservoir above the outlet reservoir. **(B)** Cross-section of the microfluidic perfusion chamber along the red dashed line shown in **A** (drawn not to scale). Neutrophil is shown as a red sphere and P-selectin molecules on the cover slip are shown in blue. Only one of the nine perfusion chambers is drawn.

Fig. 3.

Dual color footprints of a rolling neutrophil. Footprints of a rolling neutrophil from a wild type mouse stained with Vybrant DiO and DiI at indicated times. Top row, dual color footprints; middle row, DiO footprints; and bottom row, DiI footprints. Rolling is from left to right. Wall shear stress 8 dyn/cm². P-selectin 20 molecules/μm². Scale bar, 5 μm.

Fig. 4.

DqDF is independent of the spectral properties but dependent on the cellular location of the fluorochromes. The DiO and DiI single-color qDF footprints at $t = 0.3$ s in Fig. 3 were used to generate separate 3D reconstructions for (A) DiO and (B) DiI, respectively. The DiO and DiI qDF footprints used for 3D-reconstruction are also shown in insets. Rolling is from left to right. Wall shear stress 8 dyn/cm^2 . P-selectin $20 \text{ molecules}/\mu\text{m}^2$.

Fig. 5.

DiO and DiI reveal very similar microvilli coordinates in the footprint of a rolling neutrophil. The x , y , and z coordinates of the microvilli tips were extracted from Fig. 4A and B and plotted as **(A)** and **(B)**, respectively, with the z -coordinates of microvilli tips shown by colors of the data points according to the color coding tables on the right. Data points at $x < -4 \mu\text{m}$ represent tether anchorage points. Black arrow denotes the direction of rolling. P-selectin $20 \text{ molecules}/\mu\text{m}^2$. Wall shear stress $8 \text{ dyn}/\text{cm}^2$.

Fig. 6.

(A) DiI fluorescence intensity plotted as a function of the DiO fluorescence intensity for every pixel in the DqDF image at $t = 0$ s in Fig. 3 (DiO-DiI cell 1). The red line represents linear fit. (B-C) The residuals of the linear fit plotted as a function of the DiO fluorescence intensity for DiO-DiI cell 1 (B) and DiO-DiI cell 2 (C). The red line represents a residual value of zero. Vertical blue dashed lines separate the three intervals containing DiI residuals for pixels with DiO intensity less than 500, between 500 and 1000, and greater than 1000, respectively. (D) The DqDF image at $t = 0$ s in Fig. 3. Bar is 5 μm . The representative structure used for object-based analysis is shown by white dashed square. (E) The true overlap distance of normalized DiI and DiO intensity distribution of the representative structure enclosed by dashed square in D (magnified image shown in inset; white horizontal bar 300 nm). The vector is shown as blue line.

Fig. 7.

DqDF reveals localization of mCherry-paxillin relative to the membrane topography. **(A)** Dual-color DqDF image (yellow) generated by superimposing two single-color qDF images showing distribution of DiO (green) in the membrane and mCherry-paxillin (red) in the cytosol, respectively. **(B)** The DiO qDF image was processed to generate a 3D reconstruction of the cell footprint showing x and z coordinates of individual microvilli in the footprint. **(C)** The 3D-footprint in **B** rotated clockwise about the x -axis by 90° and the mCherry-paxillin qDF image overlaid to reveal the localization of mCherry-paxillin (red) relative to membrane topography (grey, cell body vs. microvilli). Rolling is from left to right. P-selectin 20 molecules/ μm^2 . Wall shear stress 8 dyn/cm². Scale bar 5 μm .

Fig. 8.

(A) The distributions of average mCherry intensities for single isolated microvilli ($n = 16$) and those within interconnected ridges ($n = 38$). The flat bars represent medians of the two distributions. Two headed arrow represents a $P \ll 0.01$. (B-C) Residuals of the linear fit plotted as a function of the DiO fluorescence intensity for the DiO-mCherry cell 1 (B) and cell 2 (C). Red line represents the residual value of zero. Vertical blue dashed lines separate the three intervals containing mCherry residuals of pixels with DiO intensity less than 75, between 75 and 150, and greater than 150, respectively. *, different from zero ($P < 10^{-8}$).

Reviewer Fig. 1.

Intensity correlation plots for DiO-mCherry cell 1 (**A**) and cell 2 (**B**) with the mCherry and DiO axis set to the same scale.

Table 1
Parameters and statistics of the linear correlation

The equation of the straight line is $y = a + bx$ [41]. a , y intercept; b , slope; σ_a , uncertainty in a ; σ_b , uncertainty in b ; $\text{Cov}(a,b)$, covariance of a and b ; r_{ab} , coefficient of correlation between the uncertainty in a and the uncertainty in b . PC, Pearsons' coefficient [4]; P -value, statistical significance of PC [11]. Positive or negative value of r_{ab} relates to same or opposite signs of α_a and σ_b , respectively.

Cell No.	a	b	σ_a	$\sigma_b \times 10^5$	$\text{Cov}(a,b) \times 10^8$	r_{ab}	PC	P -value
DfO-Dil								
Cell 1	-0.8323	0.7116	0.0140	9.4865	-5.3152	-0.3930	0.98	100 %
Cell 2	-0.7457	0.6931	0.0186	12.095	-4.7711	-0.3736	0.98	100 %
DfO-mCherry								
Cell 1	6.3507	0.1502	0.0028	5.7601	-65.958	-0.6663	0.88	100%
Cell 2	7.3077	0.1475	0.0168	36.813	-51.145	-0.6258	0.87	100%