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APPENDIX

Materials and Methods

UV Visible Spectroscopy

To determine the optimal wavelengths for exciting the fluorophores used in this study, we performed absorbance measurements on dye samples dissolved in PBS (pH 7.2) using a Biotrace model 752 spectrophotometer. We scanned the near UV and visible spectrum (200-700nm interval, 5nm steps) and subtracted the absorbance values obtained from a solvent blank. We did this in triplicate to eliminate any possible random variations caused by electronic noise from the equipment.

Analytical Chromatography

In an attempt to isolate Pyronin Y from the initial mixture of methyl green, crystal violet, and Pyronin Y, and test its fluorescence properties, we prepared a sample of the available commercial compound dissolved in 96% ethanol (5% m/v). We performed multiple chromatographic runs using cellulose as the stationary phase (Whatman paper, grade 1) and applied different running solvent systems (methanol, 100% ethanol, acetone, PBS, bidistilled H_2O), both pure and combined in different proportions, as the mobile phase. We carried out qualitative analysis (Fig. 1B) with visible light (i.e., color distribution on the paper according to the effectiveness of the solvent used to separate the 3 components of the solution) and black light (i.e., separation into fluorescent and opaque components) to identify the most suitable partition coefficients for the rational design of a preparative separation protocol.

Obtaining and Preparing Pyronin Y

Based on the behaviour of the commercial mixture against different organic solvents, aqueous and miscible mixtures, we tested two preparative separation strategies: i) a liquid-liquid partition using PBS-trichloromethane as the aqueous and organic phases, respectively; ii) a solid-liquid extraction sequence using trichloromethane, followed by acetone, and finally PBS as the extraction solvents. For both methods, the fraction of interest (trichloromethane (i) and acetone (ii)) was completely dried in a rotary evaporator, and the solids obtained were weighed and dissolved in anhydrous DMSO (Sigma, molecular biology quality) at the solubility limit (~30 mg/mL). The system was then diluted in sterile PBS (pH 7.2) until biocompatibility of the vehicle was reached at the final experimental concentration (DMSO < 0.05% v/v in culture). The Pyronin Y fractions thus prepared were stored in the dark at 4°C in a refrigerator where they remain stable for at least 6 months. We estimated the purity of the preparations by UV-visible (250-700nm), spectroscopy, and chromatography (Whatman, grade 1 and TLC).

PMN Purification

The PMN purification process begins with a centrifugation step to extract the highest possible content of plasma and platelets. The sample is then resuspended in PBS (pH=7.2) at twice the original volume. A primary PMN extraction is performed using a Ficoll-Paque gradient (0.5 ml of Nycoprep solution per ml of blood). The sample is then centrifuged for 15 minutes at 500xg, and the halo of mononuclear leukocytes is removed. The buffy coat, rich in PMN and erythrocytes, is resuspended in PBS and centrifuged again for 10 minutes. Finally, sedimentation by Dextran (1% m/v) allows the separation of most of the erythrocytes. To do this, one part of buffy coat, one part of 6% Dextran, and four parts of physiological solution are mixed, inverted, and allowed to settle for 20 minutes. After this time, the phase with the lowest density (transparent reddish liquid) is extracted, diluted 50% with PBS (1X, pH 7.2), and centrifuged for 10 minutes to obtain a pellet with erythrocytes and a high PMN content. Finally, the remaining erythrocytes are lysed by resuspending the pellet in nine parts of bidistilled water for 30 seconds and stopping the lysis with one part of PBS 10X. The washing step is then repeated, and the cells are counted in a Neubauer chamber. A total of 10 peripheral blood samples were used for the present study.

Culture and Enzymatic Induction of H_2O_2

For PMN culture, adjustable acrylic trays with circular coverslips (diameter 16mm) were used, and both were sterilized using a 36-minute humid heat cycle in an electric pressure cooker and mounted under sterile conditions to prevent surface contamination. The coverslips were pretreated for 30 minutes at 37°C in a steam-saturated environment with 50µl of a 1mg/mL solution of bovine Type I collagen (Sigma), which acted as a substrate for cell adhesion mediated by integrins and other membrane adhesins. After the incubation time, the excess non-adhered collagen was removed, and the surface was incubated for 15 minutes with 50µl of blocking medium (100µl 10x M199 solution + 900µl bidistilled H2O + 110µl autologous platelet-poor plasma) to neutralize non-specific binding sites. After blocking, the solution was extracted, and neutrophil adhesion proceeded

by dispensing 50µl of a $3x10^6$ PMN/mL suspension ($1.5x10^5$ PMN/cover slips) and incubating for 40 minutes at 37°C. Once adhesion was complete, NETosis was induced with Glucose Oxidase (GO, Sigma), which catalyzed the oxidation of glucose present in the culture medium to form D-glucono-δ-lactone and hydrogen peroxide (H_2O_2). The suspension with non-adherent cells was removed, and 50µl of inducing solution (1mL blocking medium + 4µl GO 1UI/mL, prepared immediately before starting the stimulation) or control medium was added and incubated for 150 minutes, after which 200µl of cold 4% PFA was gently added to fix the sample overnight at 4°C or for 15 minutes at room temperature.

Staining and Mounting

The fixed cells were stained in the culture tank by adding 20μl of each dye(s) alone or combined (purified from Pyronin Y = 1μg/ml; aqueous phase of commercial reagent extracted with $CHCl_3$ (MG:PyY≈1:1) = 5μg/ml; Evan's blue = 10μg/ml). After a 10-minute incubation at RT in a humid chamber, the excess dye was removed and washed gently along the walls of the container to avoid accidental mechanical removal of cells and/or NETs with 200μl of PBS. Finally, the coverslips were removed from the culture chambers and mounted on slides using 10x glycerol:PBS (9:1, pH 8) as the mounting medium and transparent nail polish to seal the coverslip to the slide.

LSM 880 System Configuration

The LSM880 multimodal confocal microscopy workstation (Zeiss, Germany) installed at LAMAE (IBB-UNER-CONICET, FING) was used in all microscopy trials. The equipment has 7 excitation lines provided by a multibeam Argon gas laser (458nm, 488nm, and 514nm, 25mW), a red HeNe laser (633nm, 5mW), a green solid-state-pumped diode (DPSS, 561-10nm, 20mW), and 2 violet pulsed diode lasers (405nm, 440nm, 30mW) that are necessary for FLIM. Additionally, the equipment has an array of 3 photodetectors that allow multispectral analysis of fluorescence emission (3-channel QUASAR detection system, Zeiss), a 32-channel super-resolution system that allows improving XYZ resolution below the diffraction limit (Airyscan®, Zeiss), a module for FLIM acquisition (Becker & Hickl, Germany), a thermostated culture chamber with a motorized piezo stage, and 2 high-performance PCs equipped with equipment control software and digital image processing.

3D LSCM (z-stack)

Using 458, 488, 514, 561, and 633nm excitation sources, the 63X oil immersion objective (Objective Plan-Apochromat 63x/1.4 Oil DIC M27, Zeiss), and the QUASAR detection system, fluorescence optical sections or DIC along the z-axis were acquired. The generated images were combined into series of 3D images (z-stacks) in ".czi" format. Depending on the experiment, 2 or 3 channels were stored, one for DIC and the others for color. This type of acquisition was performed in 10 independent staining experiments.

Spectral LSCM (λ-stack)

Using 633nm, 440nm, and 561nm excitation sources and the same objective lens, the QUASAR detection system was set up to obtain emission spectral scans in ranges between 530 and 725nm with 5nm windows over different regions of interest (ROI) plotted on PMN nuclei. The fluorescence images acquired throughout the scan were combined into ".czi" files consisting of a series of 2D images of one channel (λ-stacks). The determinations were repeated 3 to 6 times with independent staining.

Optical Super Resolution (SR-LSCM)

Using the 561nm excitation source, 63X objective, and AiryScan detection module, super-optical (140nm) resolution fluorescence z-stacks (BP 595/50) were obtained, processed, and stored. This kind of acquisition was repeated in 4 independent experiments.

Fluorescence Recovery Post Photobleaching (FRAP)

Using the 440nm pulsed diode laser at 100% power, circular areas of PMN nuclear lobes were irradiated for 1 minute, and the loss and recovery of fluorescence in the irradiated area were recorded at 5-second intervals using the 561nm laser and the detection band of 595/50nm. The FRAP experiment was repeated 4 times in each of the 3 independently stained samples.

Fluorescence Lifetime Micrograph (FLIM)

Using the 440nm pulsed diode laser at 4% power and the 500-550nm detection channel (Channel 2), the photons emitted by the pixels of the region of interest (time-domain FLIM) were collected for 2 minutes. For the adjustment of the decay curves, a double exponential model was assumed, and the criterion $0.8 \leq \chi^2 \leq 1.3$ was set for the goodness of fit. The raw data were stored in «.sdt" files and processed with the SPCImage NG Data Analysis software (Becker & Hickl, Germany) to obtain the interactive phasor diagrams.

Data Analysis and Image Processing

The spectroscopic and fluorescence intensity data were processed and plotted with R and Excel (Microsoft). LSCM

images were acquired and eventually digitally processed with ZEN 2.3 software (Zeiss, Germany). The quantitative analysis of intensities on LSCM images was performed with FIJI (Image J-NCBI). FLIM images were acquired and processed with the SPCM and SPCImage NG Data Analysis software, respectively (Becker & Hickl, Germany).