Evaluation of chemo and photo-toxicity of a live fluorescent dye for cell analysis

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Abstract

Live cell imaging is used to track the dynamic adaptation of cell size and motility to various external factors. Bright field configuration can be used for these experiments, however the analysis can be challenging and difficult to automate. In this direction, a superior alternative is represented by the use of live-cell dyes, which provide intense fluorescence from subcellular structures of living cells. Yet, the potential chemo- and photo-toxicity of the fluorophores poses the necessity of an accurate protocol optimization to avoid artefacts. Toxicity studies generally focus on cell proliferation and apoptosis, neglecting the cellular activities under investigation. Here, we present the case of SYTO 13 in combination with primary endothelial cells. The optimization of the staining procedure is tested comparing cell proliferation and motility rate. In addition, the combined effect of staining and fluorescent illumination, reporting for photochemical toxicity, is evaluated. We demonstrate that while cell viability and proliferation are mainly unaffected by the staining and imagining protocols, a significant reduction of the motility rate is induced both by the chemical dye alone and in combination with fluorescent illumination. The general implications for this procedure are discussed.

Introduction

Live cell imaging is widely used to track the dynamic adaptation of cell size, shape and motility to various external factors, including gradients of soluble molecules [1], substrate topography [2], rigidity [3] and density of adhesion points [4], flow-generated wall shear stress [5], and others. Imaging in bright field configuration can be used for some of these experiments allowing for long-term acquisition without photo-toxicity and thus illumination-related artefacts. However, the analysis of the resulting time-lapses can be challenging and difficult to automate due to the limited contrast and absence of 3D information [6]. One possible alternative is represented by the use of commercial live-cell dyes, which are added to the cell medium and provide intense fluorescence from the membrane, different organelles or DNA/RNA of living cells, yielding bright signal for cell tracking, segmentation, and rendering.

The potential chemo- and photo-toxicity of the fluorophores poses however, the necessity of an accurate protocol optimization in order to avoid artefacts introduced during the staining procedure or upon imaging. Standard toxicity studies are generally limited to the evaluation of cell proliferation and apoptosis, neglecting the more subtle, but significant, metabolic alterations which can affect the cellular activities under investigation [7].

Here, we establish and validate a twofold analysis of the chemo and photo-toxicity of fluorescent dye towards its use in cell motility assays. The method can be applied to calibrate the dye application in terms of optimal working concentration in protocols for staining and illumination. We present the case of SYTO 13, a commonly adopted chemical fluorophore [8, 9], in combination with primary human endothelial cells (HUVECs). The motility behaviour of these cells is relevant to study their response to flow and wall shear stress, subtending to their function in the maintenance of tissue homeostasis [10]. In addition, these cells are selected for their high sensitivity to noxious environmental stimuli. The concentration-dependent effect of the staining procedure is tested comparing cell proliferation and motility rate. In addition, the combined effect of staining and fluorescent illumination, reporting for photochemical toxicity, is reported.

The same evaluation procedure can be extended to other dyes and cell types, with specific adaptations, therefore rendering the present method a valid preparation for general live cell fluorescent analysis.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs; ThermoFisher Scientific, USA) were grown in medium M200PRF supplemented with fetal bovine serum 20% v/v, hydrocortisone 1 mg ml^{-1}, human epidermal growth factor 10 ng ml^{-1}, basic fibroblast growth factor 3 ng ml^{-1}, and heparin 10 mg ml^{-1} (all reagents from ThermoFisher Scientific) and maintained at 37°C and 5% CO_2. All reported experiments were performed using cells with less than 7 passages in vitro. Surfaces for experiments were coated with glutaraldehyde cross-linked gelatin as previously reported [11].

Cell Microscopy

An inverted Nikon-Ti spinning disk confocal microscope (Nikon, Japan) equipped with an Andor DU-888 camera (Oxford Instruments, United Kingdom) and a pE-100 LED illumination system (CoolLED Ltd, Andover, United Kingdom) was used to perform both proliferation and motility assays. Images were acquired with a 20×, 0.75 NA short-distance air objective (Plan Apo, Nikon, Japan).

Cell live stains

The cell-permeant SYTO 13 green fluorescent nucleic acid stain (Thermofisher Scientific) was used according to the manufacturer's instruction. The following concentrations were tested: 0.01 μM, 0.05 μM, 0.1 μM, 0.35 μM, 0.5 μM, 1 μM, 1.4 μM, 2 μM, 3 μM, 4 μM, 5 μM. Untreated cells and cells in which just the vehicle (DMSO) were included as control.

Proliferation Assay

Cells were seeded at 80 cells/mm^2 density and allowed to recover overnight in culture. Samples were then incubated with SYTO 13 diluted in full medium to reach the test concentrations for 30 min, after which the solution was replaced with fresh culture medium. 24 h later samples were fixed in 4% paraformaldehyde and immunostained for Ki67.

Specifically, cells were permeabilized for 10 min with 0.1% Triton-X100 in PBS at room temperature (RT). They were incubated with 5% bovine serum albumin (BSA) in PBS for 1 h at RT. The samples were then incubated with Ki67 primary antibody (1:500, rabbit polyclonal, Abcam) overnight at 4°C. Subsequently, the samples were rinsed with 5% BSA in PBS and then incubated with the secondary antibody (1:200, donkey anti-rabbit Alexa-555, ThermoFisher

Scientific) and DAPI (1:1000, ThermoFisher Scientific) for 45 min at RT. The percentage of proliferative cells was determined by quantification of Ki67-positive cells and comparison with the total number of cells revealed by the DAPI staining.

Motility Assay

Cells were seeded at an initial density of 40 cells/mm^2. Samples were then incubated with SYTO 13 diluted in full medium to reach the test concentrations for 30 min, after which the solution was replaced with fresh culture medium. The time-lapse experiments were set to acquire images for multiple positions with a time resolution of 10 min for a total of 6 h. During imaging, the microscope incubator chamber (Life Imaging Services, Switzerland) was set at 37°C, 5% CO_2 and 90% humidity. Two illumination conditions were adopted. For the evaluation of chemo-toxicity, a single bright field (BF) channel was selected (i.e. no fluorescent illumination). For the evaluation of the combined chemo and photo-toxicity, cells were imaged in parallel in two separate BF and fluorescence channels. SYTO 13 fluorescence was excited at $\lambda_{excitation}$ = 440 nm and collected at $\lambda_{emission}$ = 550 nm, using a FITC filter cube (Nikon). The applied exposure time and illumination intensity yielded an energy density of 9.28 kJ/m^2.

Image Analysis

The analysis of cell motility was performed with ImageJ (National Institute of Health, US) and consisted of two sequential parts. First, the pre-processing used the 'contrast limited adaptive histogram equalization', the 'background removal' and the 'contrast adjustment' plugins of ImageJ to improve individual cell detection. Second, the cell tracking used the 'Manual Tracking' plugin of ImageJ. Specifically, the cell barycentre was detected at each time of measure to obtain a migration track. The total travelled distance over 6 h was computed as the distance between the cell position at time 0 and 6 h.

IC$_{50}$ determination

The IC$_{50}$ is defined as the concentration of an inhibitor reducing the response under analysis to half of its maximal value. IC$_{50}$ concentrations for the inhibition of cell proliferation and motility were obtained fitting the corresponding data points with a dose-response curve [12]. In particular, the experimental data on proliferation rate and total travelled distance under bright field illumination were fitted using a simple dose-response curve as described in Hill model.

Eq. 01:

$$y(x) = A1 + \frac{A2 - A1}{1 + 10^{(\log IC_{50} - \log x)h}}$$

Where *A1* and *A2* are the bottom and top plateaus of the curve, in the same units as the concentration. IC_{50} is the concentration that gives half-maximal inhibitory effect in the same units as the response. *h* is the Hill slope factor.

Experimental data of total travelled distance obtained under bright field and fluorescent illumination were fitted using a multiphasic dose-response model to consider both chemo and photo-toxicity.

Eq. 02:

$$y(x) = A1 + (A2 - A1)\left[\frac{p}{1 + 10^{(\log IC_{50}1 - \log x)h_1}} + \frac{1 - p}{1 + 10^{(\log IC_{50}2 - \log x)h_2}} \right]$$

Where *A1* and *A2* are the bottom and top plateaus of the curve, in the same units as the concentration. $IC_{50}1$ and $IC_{50}2$ are the midpoint potency parameters for the two different phases, respectively, while h_1 and h_2 are the corresponding Hill slope factors. *p* is the fraction of the curve comprising the more potent phase.

For the fitting, *A1* and *A2* values were inserted manually: with *A1* equal to zero corresponding to no motility and no proliferation and *A2* set to the value measured in the corresponding control condition (i.e. the treatment with DMSO).

Statistical Analysis

Data were analysed, tested for statistical significance, fitted, and visualized using OriginPro 2020b (OriginLab, Northampton, Massachusetts, USA). The total number of independent experiments is indicated in each figure caption. The Shapiro–Wilk test was used to test for normality of data. Tukey's multiple comparisons test was used to analyse statistical significance between data.

Results

The mean proliferation rate for HUVECs incubated with DMSO, at the highest tested concentration (see materials and methods), was not significantly different than the one measured in the untreated control group (Figure 1A). Specifically, the mean percentages of proliferative cells for the two groups were 48.02 ± 6.12% and 49.18 ± 6.24% for the control (n = 393) and DMSO treated group (n = 294), respectively. The travelled distance over 6 h, informing over the rate of cell motility, was similarly not different in control and DMSO treated cells (Figure 1B) for all tested illumination conditions. In particular, the values measured under bright field (BF) illumination were 250.68 ± 10.55 μm and 240 ± 24.74 μm for the control (n = 75) and DMSO treated group (n = 53), respectively. Parallel illumination with fluorescent light did not alter these values, yielding travelled distances of 267.86 ± 18.45 μm and 253.87 ± 19.30 μm, for the control (n = 60) and DMSO treated group (n = 47), respectively. Altogether, these results confirmed that DMSO is not significantly altering the proliferative or motility rate of HUVECs. In addition, they demonstrated that the selected fluorescence illumination conditions do not introduce, *per se*, any detectable photo-toxicity in the cell system under analysis.

The effect of the SYTO 13 staining on the proliferation rate of HUVECs was analysed over a wide range of concentrations that included the values recommended by the manufacturer (from 0.01 to 5 μM). In particular, treatment with concentrations up to 1.4 μM had no measurable effect on cell proliferation (Figure 2). The proliferation rate decreased exponentially upon treatment with 2 μM of SYTO 13 and higher. At the highest tested concentration of 5 μM the residual proliferation rate was of about half of the control value (49.18 ± 6.24%; Figure 2A). Fitting of the data for the SYTO 13 inhibitory effect on cell proliferation individuated a corresponding IC_{50} value of 3.89 μM.

Next, the effect of microscopy illumination on the motility of SYTO 13-treated HUVECs was analysed measuring the total travelled distance over a period of 6 h (Figure 3). To decouple potential chemo and photo-toxic effects of the fluorescent dye, two experimental conditions were compared. Simple BF illumination did not affect cell motility in cells treated with SYTO 13 concentrations lower than 3 μM. Above this value, the travelled distance was drastically reduced and cell motility was completely inhibited at the highest tested concentration (Figure 3A). Fitting of these data for the inhibitory effect on cell motility under simple BF illumination individuated a corresponding IC_{50} value of 2.88 μM.

Addition of fluorescence to the illumination protocol (BF-FLUO) caused a significant reduction of cell motility at all SYTO 13 concentrations, with the exception of the lowest tested value (0.01 μM). At concentrations of 3 μM or higher, cell motility completely ceased (Figure 1A). The data are well fitted by a bi-phasic dose-response curve (see materials and methods) individuating two IC_{50} values at 0.07 and 2.04 μM, respectively. Altogether, these data demonstrate that the treatment of HUVECs with SYTO 13 introduces a concentration-dependent inhibition affecting both cell motility and proliferation. Fluorescent illumination exacerbates this effect, which becomes significant at much lower concentrations.

Discussion.

This work establishes a simple sequence of assays to evaluate the toxicity of fluorescent dyes used for the live tracking of cell morphology and motility. While most of the reported toxicity data for such compounds are limited to a proliferation or live/dead assay, we demonstrate that more subtle artefacts can alter the values measured for complex cellular activities, which entail adhesion to the substrate and force generation necessary to promote cell motility.

The specific case of SYTO 13 is presented in combination with a subconfluent culture of primary human endothelial cells (HUVECs). These cells represent a well-established model for endothelial differentiation and homeostasis [13]. In addition, a growing interest exists in the generation of confluent endothelial monolayers at the luminal interface of cardiovascular devices, in the protective process of endothelialization [14]. The validation of endothelialization strategies necessarily requires the study of cell interaction with artificial substrates *in vitro*. Since primary cells retain their endothelial characteristics for a limited number of passages *in vitro*, the natural choice for live cell studies is the use of chemical dyes that, once added to the cell culture, render the cell membrane, organelles or nucleus intensely fluorescent. A widely used compound for live cell staining is SYTO 13 [9, 15, 16]. However, its viability in combination with endothelial cells has not yet been reported.

When assessing the division rate of the cells treated with SYTO 13 at the recommended concentrations it is indeed evident that there exists only a non-significant effect on cell proliferation (Figures 1 and 2), and based purely on this aspect the conclusion would be that the dye is not toxic. However, a deeper investigation detecting alterations in the cell movement upon illumination changes completely the picture (Figure 3). Here, SYTO 13 shows both a clear chemo-toxicity, reducing the cell motility, and an even stronger combined chemo- and photo-toxicity resulting in impaired migration upon fluorescence excitation of the dye even at the lowest tested concentrations (Figure 3). These conclusions are supported by the quantitative extrapolation of the half maximal inhibitory concentration (IC_{50}) for the various tested experimental configurations. The IC_{50} for SYTO 13 inhibitory effect on proliferation (Figure 2) is in fact more than one order of magnitude higher of the corresponding value for motility (Figure 3).

Altogether, these data provide a clear demonstration of the importance of such thorough evaluation. Similar calibration schemes can be adapted and optimized for other fluorescent dyes and cell types, in keeping with the main passages reported here.
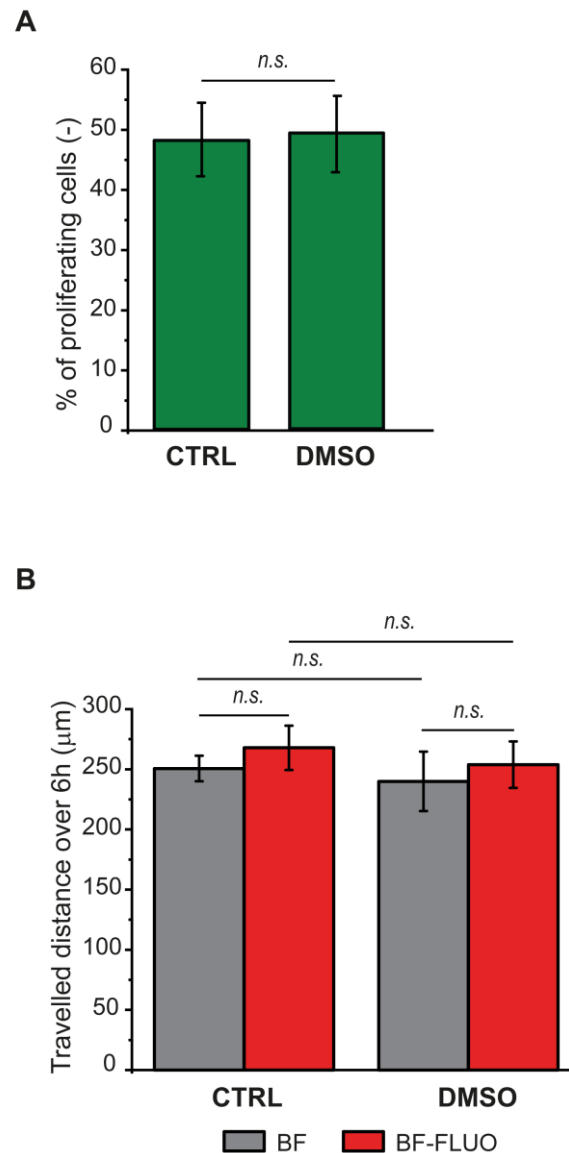
Figures

Figure 1: Treatment with DMSO does not affect proliferation or motility of HUVECs. **A**) Percentage of proliferating cells in control (CTRL) and DMSO-treated cells. **B**) Travelled distance (over a total period of 6 h) in control (CTRL) and DMSO-treated cells under bright field illumination (BF; grey histograms) or bright field and fluorescence illumination (BF-FLUO; red histograms). Data are reported as mean ± standard deviation of 3 independent experiments. No significant difference between the population means is indicated (n.s.).
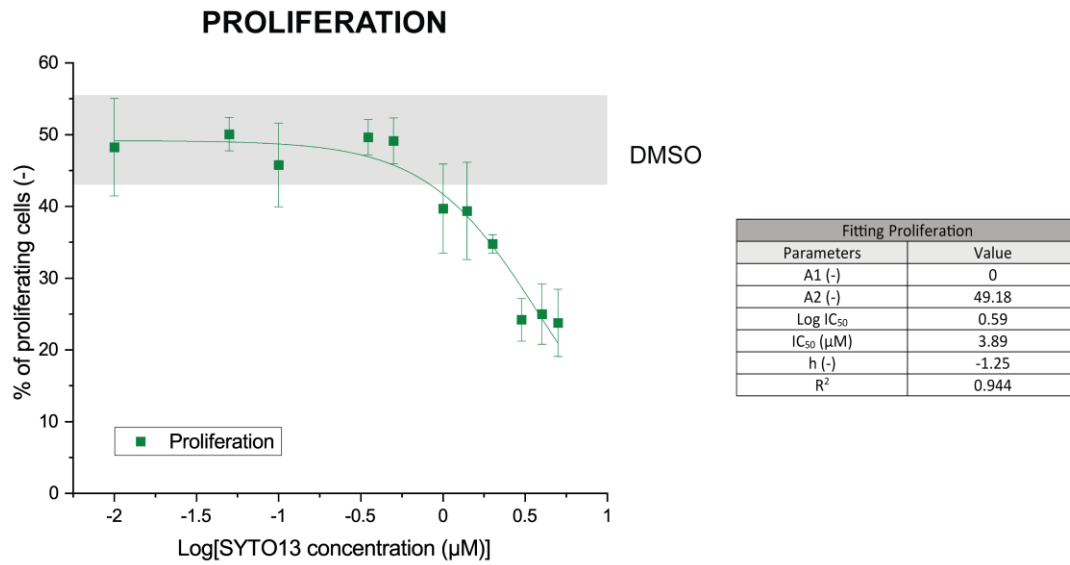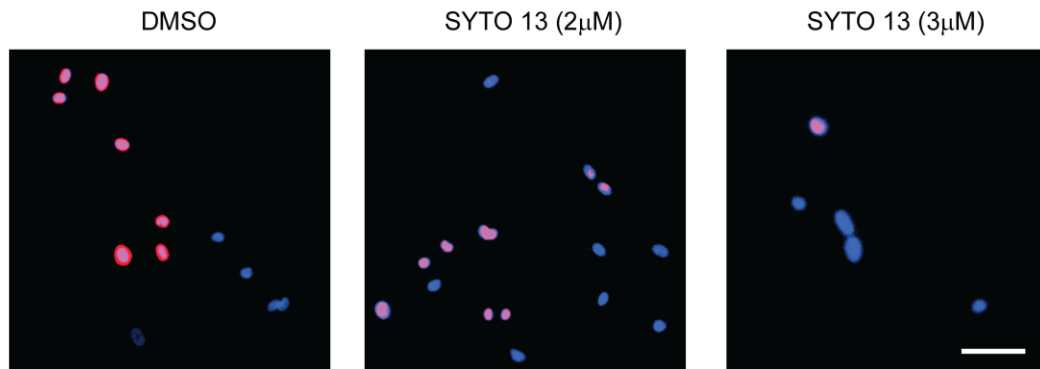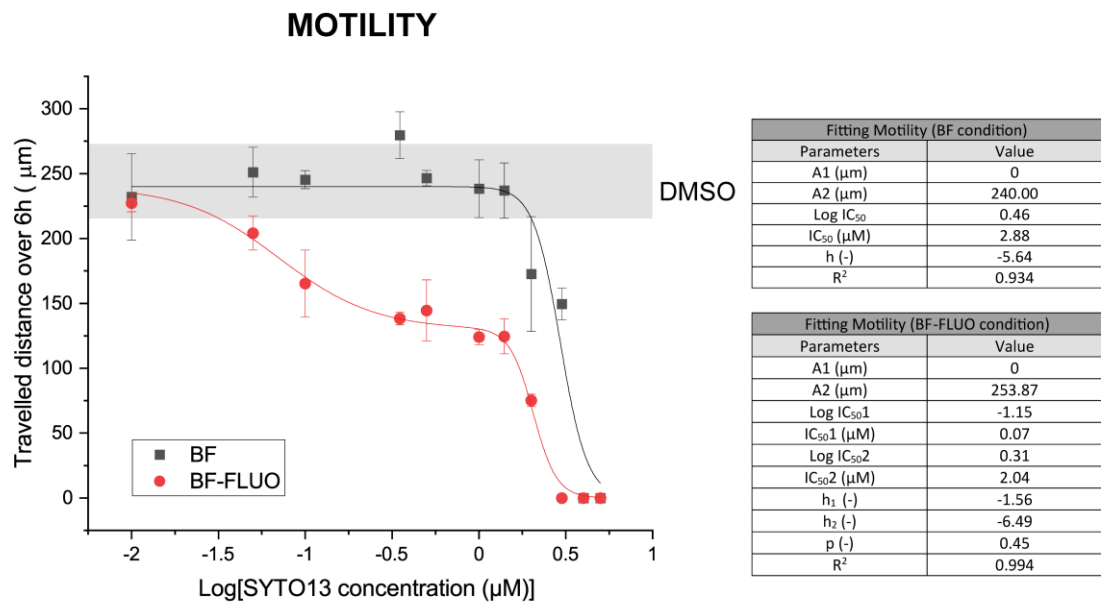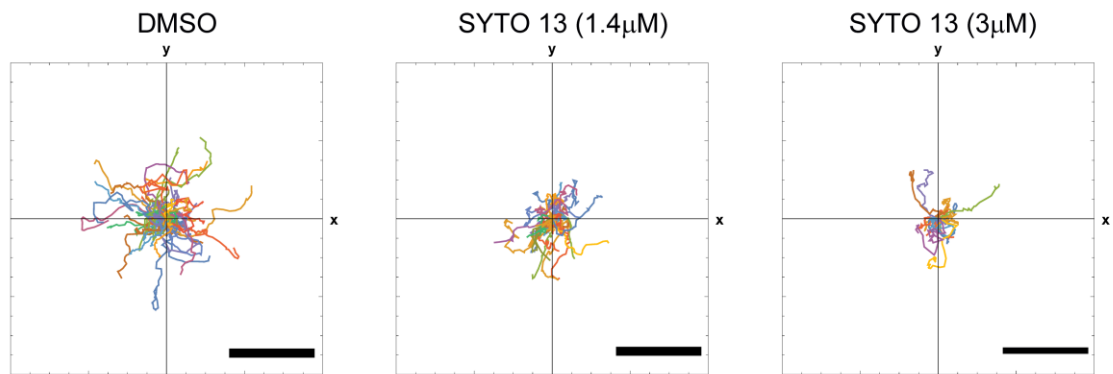
A

PROLIFERATION

Fitting Proliferation	
Parameters	Value
A1 (-)	0
A2 (-)	49.18
Log IC$_{50}$	0.59
IC$_{50}$ (µM)	3.89
h (-)	-1.25
R^2	0.944

B

Figure 2: SYTO 13 dose dependent inhibition of HUVECs proliferation. **A**) Percentage of proliferating cells upon treatment with increasing concentrations of SYTO 13 (from 0.01 to 5 µM; left). Experimental data (solid green squares) are expressed as mean ± standard deviation of 3 independent experiments. A grey bar indicates the values measured in the DMSO control. A solid green line individuates the fitting curve. The corresponding fitting parameters are reported in the table (right). **B**) Corresponding immunofluorescence images reporting the fraction of Ki67 positive (red nuclei) and negative (blue nuclei) cells for DMSO and SYTO 13 treated samples. Scale bar is 50 µm.

A

Fitting Motility (BF condition)	
Parameters	Value
A1 (μm)	0
A2 (μm)	240.00
Log IC$_{50}$	0.46
IC$_{50}$ (μM)	2.88
h (-)	-5.64
R^2	0.934

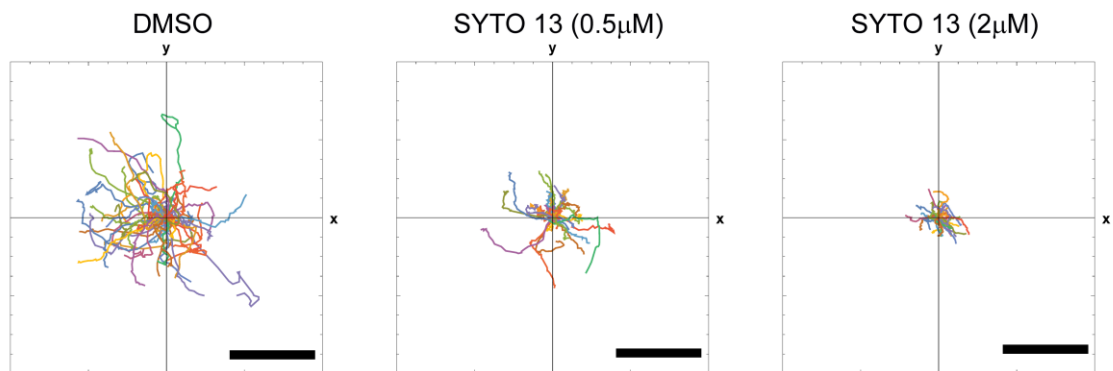| Fitting Motility (BF-FLUO condition)	
Parameters	Value
A1 (μm)	0
A2 (μm)	253.87
Log IC$_{50}$1	-1.15
IC$_{50}$1 (μM)	0.07
Log IC$_{50}$2	0.31
IC$_{50}$2 (μM)	2.04
h$_1$ (-)	-1.56
h$_2$ (-)	-6.49
p (-)	0.45
R^2	0.994

B

C

Figure 3: SYTO 13 dose dependent inhibition of HUVECs motility. **A)** The total travelled distance of migrating cells (over 6 h) upon treatment with increasing concentrations of SYTO 13 (from 0.01 to 5 μM; left). Experimental data obtained under bright field illumination (black squares) or bright field and fluorescence illumination (red circles) are expressed as mean ± standard deviation of 3 independent experiments. A grey bar indicates the values measured in the DMSO control. Solid lines individuate the fitting curves. The corresponding fitting parameters are reported in the table (right). **B-C)** Corresponding tracks of individual cell

migration upon treatment with DMSO or SYTO 13 under bright field illumination alone (B) or combined bright field and fluorescence illumination (C). Scale bar is 200 μm.

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