SUPPLEMENTARY INFORMATION:

Two-photon fluorescence anisotropy imaging in live cells allows measurement of drug target engagement.

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SUPPLEMENTARY DATA

Supplementary Data 1. One zip file (Vinegoni-PCU) containing the STL file for the assembly of the polarization control unit.
Supplementary Data 2. One zip file (Vinegoni-3Dprint.zip) containing the STL files for the 3D printing and drawing of the polarization filter cube PFC.
Supplementary Data 3. One zip file (Vinegoni-Software.zip) containing the software for the OnLine and OffLine elaboration and visualization of the fluorescence anisotropy images, and for calculating the background noise subtraction and average anisotropy: OnLine.exe, OffLine.exe, BackgroundEstimation.exe, AnisotropyCalculation.exe.
Supplementary Data 4. One zip file (Vinegoni-Dataset.zip) containing a longitudinal dataset acquired in a perfusion chamber during drug loading and washing phase, calibration measurements for high and low values of anisotropy, and cells at different time points during loading and washing time.

SUPPLEMENTARY METHODS

Preparation of PARPi-FL

Presynthesis preparation
1| Prepare all reagents and solvents needed for the synthesis. Let the BODIPY-FL NHS ester and PARP precursor warm to room temperature for 10 min.
CRITICAL Note reactants should be stored at -20 °C.
2| Open the container of extra dry acetonitrile by unscrewing the lid and attached a nitrogen-filled balloon with a needle through the septum of the container.
Synthesis of PARPi-FL
3| Set up an amber glass vial (4 mL) equipped with a magnetic stir bar on a magnetic stirrer.
4| Weigh PARP precursor (9.4 mg, 25.6 mmol) and transfer the PARP precursor to the amber glass vial.
5| Add extra dry acetonitrile (500 µL) to the glass vial containing the PARP precursor through syringe and stir for 4-5 min at 20-25 °C.
6| Add extra dry acetonitrile (500 µL) to the vendor's vial of BODIPY-FL NHS ester (5.0 mg, 12.8 mmol), close the vial and shake the vial until a homogeneous solution of BODIPY-Fl NHS ester is obtained.
7| Add slowly the solution of BODIPY-FL NHS ester (500 µL, 5.0 mg, 12.8 mmol) through a Transferpette (100-1000 µL) to the stirring solution of PARP precursor. Repeat step 6 and 7 twice to transfer residual BODIPY-FL NHS ester.
8| Add slowly triethylamine (4.6 µL) to the reaction mixture.
9| Close the reaction vial with the lid, cover the reaction amber vial with tin foil and stir the reaction mixture at 20-25 °C for (4 h).
10| Add water (3.0 mL) to the reaction vial.
HPLC purification
11| Turn of the HPLC system by pushing the button in the front of each module.
12| Start the software LCsolution on the computer and push the button Instrument1 to operate the HPLC system.

13| Equilibrate the semi-preparative column with 5% acetonitrile (0.1% TFA) in water (0.1% TFA) for 8-10 min.
14| Load the method for semi-preparative HPLC purification of PARPi-FL. The method is described in Equipment setup section.
15| Perform a cleaning run of the HPLC system injecting water (400-500 µL) through a Hamilton syringe on to the HPLC system.
16| Load a fraction of the reaction solution (390-410 µL) through a Hamilton syringe on to the HPLC system, start the purification run, and check the automated sample collection.
17| Repeat step 16 until all of the crude reaction mixture is purified by the HPLC system and collected in the vials of the fraction collector.
18| Perform a cleaning run of the HPLC system injecting water (400-500 µL) through a Hamilton syringe on to the HPLC system.

HPLC analysis

19| Exchange the semi-preparative HPLC column with the analytical HPLC column.
20| Load the method for analytical HPLC analysis of PARPi-FL. The method is described in Equipment setup section.
21| Equilibrate the analytical column with 5% acetonitrile (0.1% TFA) in water (0.1% TFA) for 4-5 min.
22| Perform a cleaning run of the HPLC system injecting water (400-500 µL) through a Hamilton syringe on to the HPLC system.
23| Load 100 µL of the collected and purified PARPi-FL solution of the first vial through a Hamilton syringe on to the HPLC.
24| Repeat step 23 for each vial containing PARPi-FL.
25| Perform a cleaning run of the HPLC system injecting water (400-500 µL) through a Hamilton syringe on to the HPLC system.
26| Weigh a self-standing centrifuge tube without the lid with Mettler balance.
27| Combine glass vials containing the HPLC purified product into a self-standing centrifuge tube.
28| Freeze dry the solvents with liquid nitrogen, place the self-standing centrifuge tube into a lyophilizer flask, put the lid on the flask and immediately attach the lyophilizer flask to the lyophilizer for 22-24 h.
29| Remove the lyophilizer flask from the lyophilizer, open the flask and take out the self-standing centrifuge tube.
30| Weigh the tube containing the product without lid with Mettler balance.
31| Take 0.9-1.1 mg of the product for the characterization and transfer it into a separate glass vial.
32| Characterize the product with analytical HPLC, LCMS, 1H-NMR, and 13C-NMR.
33| Label the tube containing final lyophilized bulk product, seal with parafilm and store at -20 °C. !Note The product can be stored at -20 °C for 12 months.

Anticipated Results

Yield
After lyophilization, pure PARPi-FL is obtained with a 70-79% yield as a red powder.
Analytical data
HPLC PDA: t_R = 14.3 min, 99.3% purity (Supplementary Fig. 1).
^{1}H-NMR (600 MHz, DMSO-d_6): δ = 12.60 (d, ^3J = 3.3 Hz, 1 H), 8.26 (dd, ^3J = 7.6, 4.2 Hz, 1 H), 8.0 (t, ^3J = 5.6 Hz, 1 H), 7.90 (t, ^3J = 7.2 Hz, 1 H), 7.82 (m, 1 H), 7.70 (d, ^3J = 4.1 Hz, 1 H), 7.46-7.41 (m, 1 H), 7.37 (t, ^3J = 6.5 Hz, 1 H), 7.24 (t, ^3J = 8.9 Hz, 1 H), 7.09 (t, ^3J = 4.6 Hz, 1 H), 6.42 (dd, ^3J = 21.5, 3.9 Hz, 1 H), 6.30 (d, ^3J = 4.5 Hz, 1 H), 4.33 (d, ^3J

= 2.9 Hz, 2 H), 3.68-3.49 (m, 4 H), 3.44-3.36 (m, 2 H), 3.22-3.13 (m, 2 H), 3.11-3.04 (m, 2 H), 2.77 (t, ^{3}J = 7.0 Hz, 1 H), 2.71 (t, ^{3}J = 7.5 Hz, 1 H), 2.46 (d, ^{3}J = 9.3 Hz, 3 H), 2.26 (d, ^{3}J = 3.5 Hz, 2 H).
^{13}C-NMR (125 MHz, DMSO-d_6): δ = 173.4, 169.6, 164.0, 159.4, 159.4, 159.1, 158.2, 158.0, 157.8, 144.8, 144.0, 134.4, 133.5, 133.0, 131.8, 131.6, 129.1, 128.9, 127.9, 126.0, 125.4, 125.3, 120.2, 117.1, 114.8, 46.5, 46.2, 44.3, 36.4, 31.2, 23.7, 14.5, 11.0.
LCMS: PDA (280 nm) t_R = 6.7 min; calculated values for $C_{34}H_{32}BF_3N_6O_3$ (MW = 640.5 g/mol): 621.3 $[M-F]^+$, 641.3 $[M+H]^+$, 663.3 $[M+Na]^+$ found values: MS(+) m/z = 621.4, 641.4, 663.3.

SUPPLEMENTARY FIGURES

Supplementary Figure 1.
PARPi-FL.
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Image Acquisition Control window of the Olympus FluoView program.
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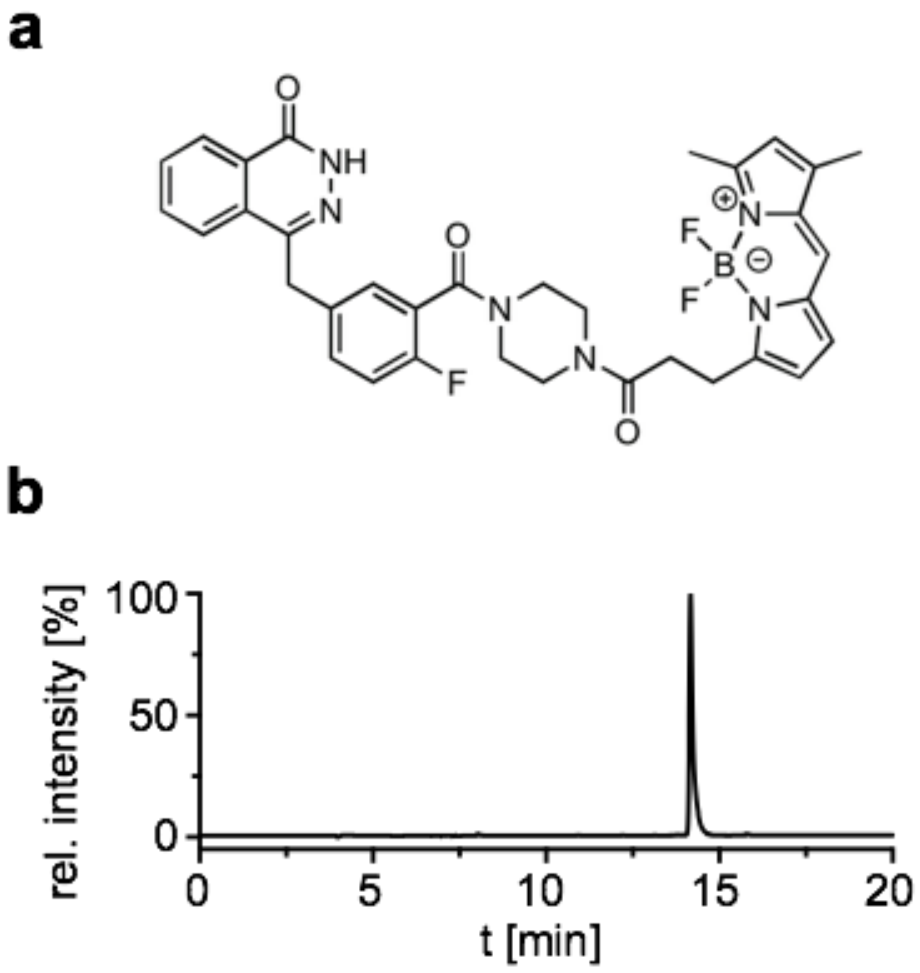
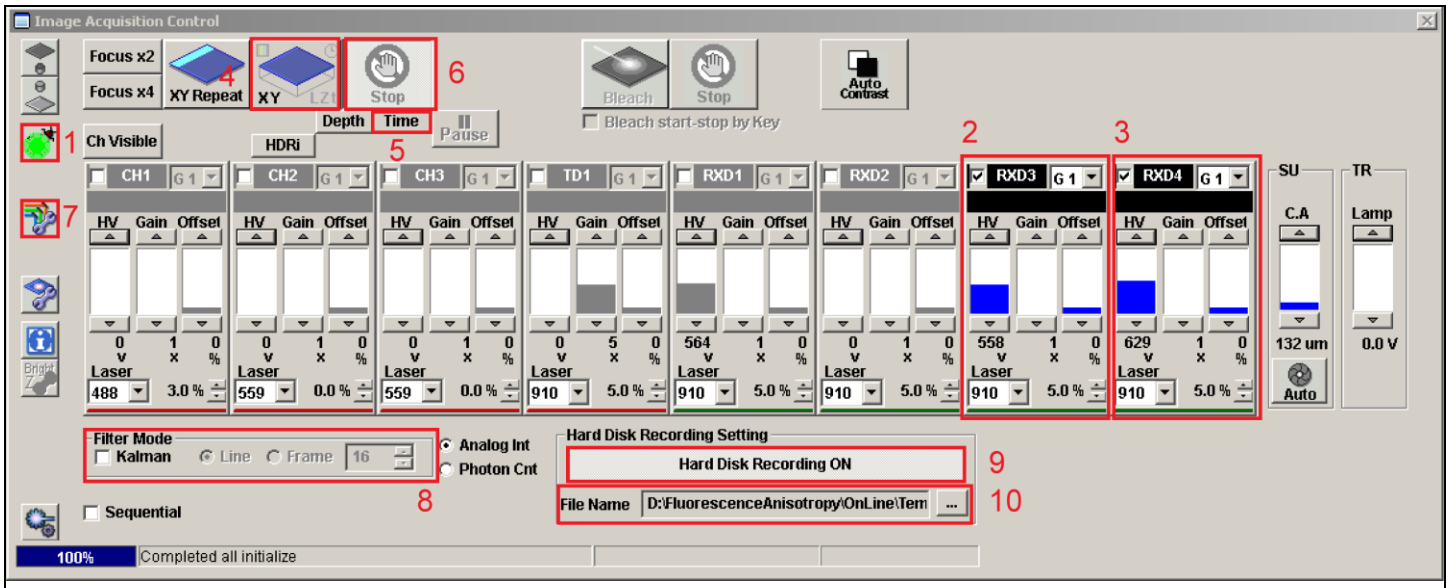


Supplementary Figure 1.

PARPi-FL.

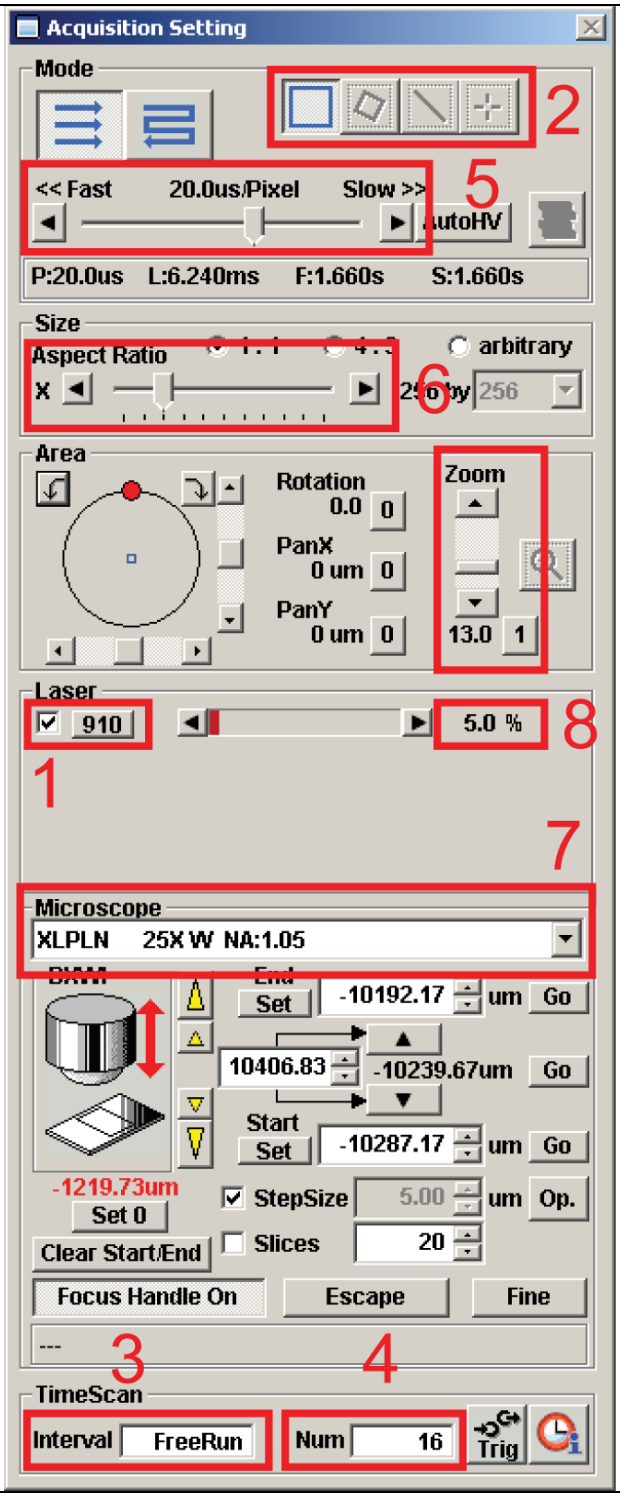
Chemical structure (a) and analytical HPLC chromatogram (b) of PARPi-FL.



Supplementary Figure 2.

Image Acquisition Control window of the Olympus FluoView program.

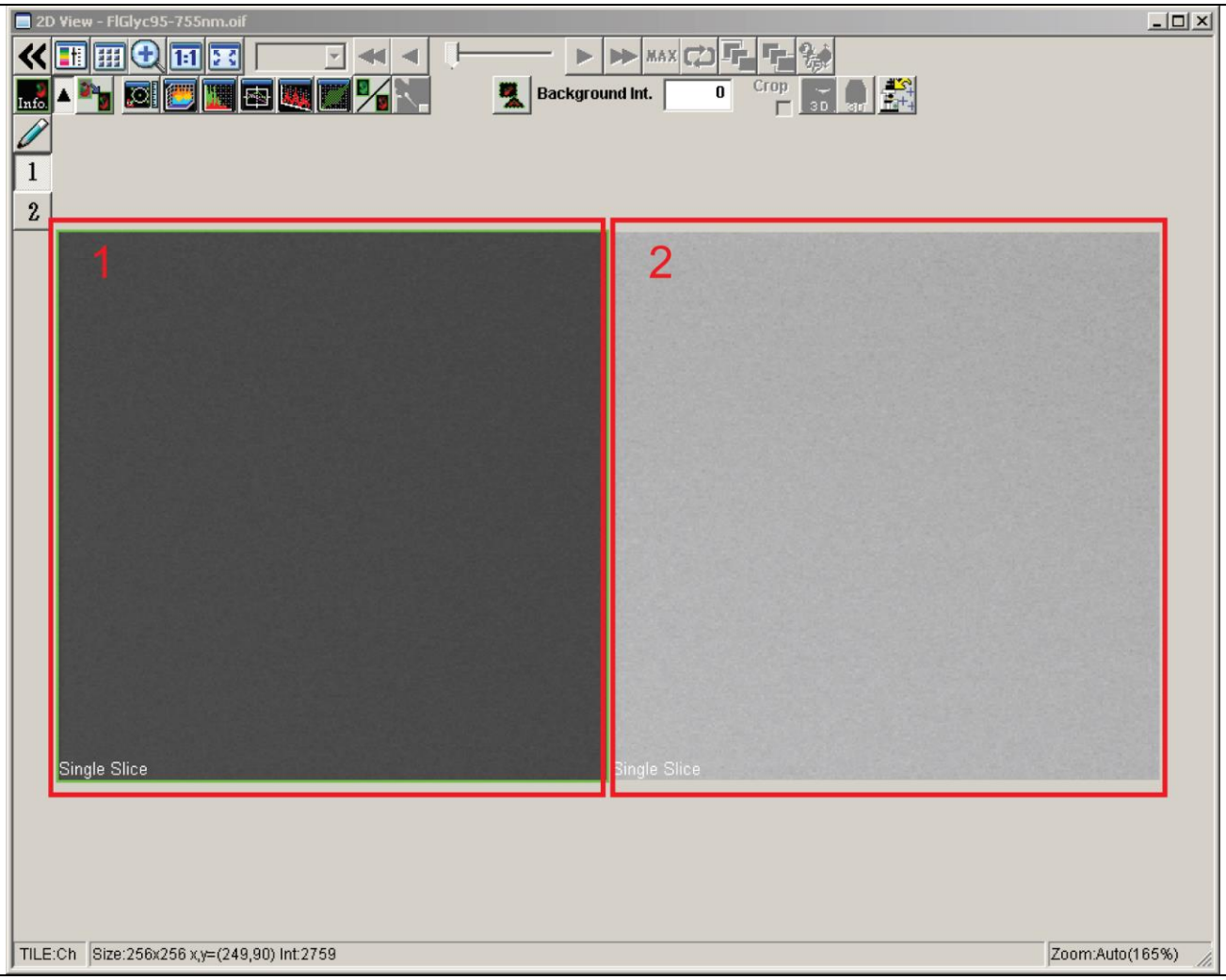
The interface allows to control the PMT voltage settings, to initiate acquisitions and image averaging.



Supplementary Figure 3.

Acquisition Setting window of the Olympus FluoView program.

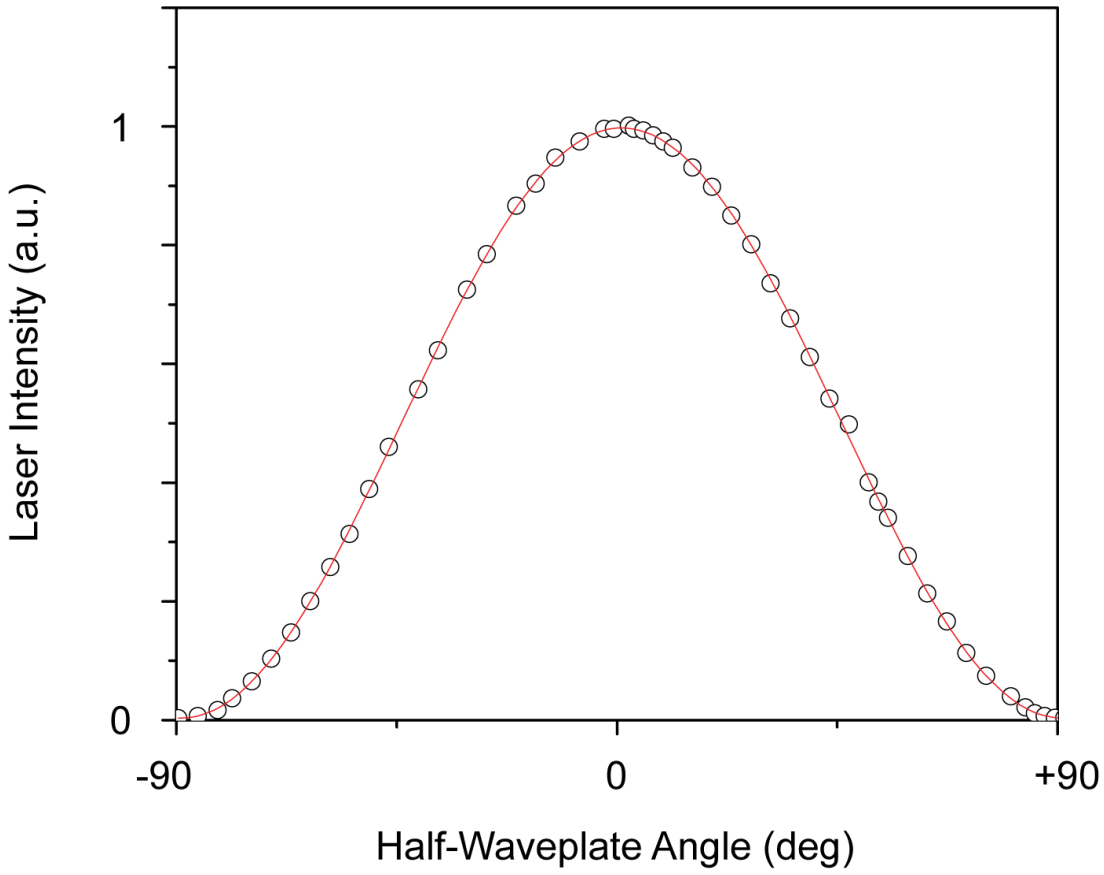
The interface allows to set image acquisition dwell time, size, zoom factor, excitation wavelength selection and laser power.



Supplementary Figure 4.

Acquired Image window of the Olympus FluoView program.

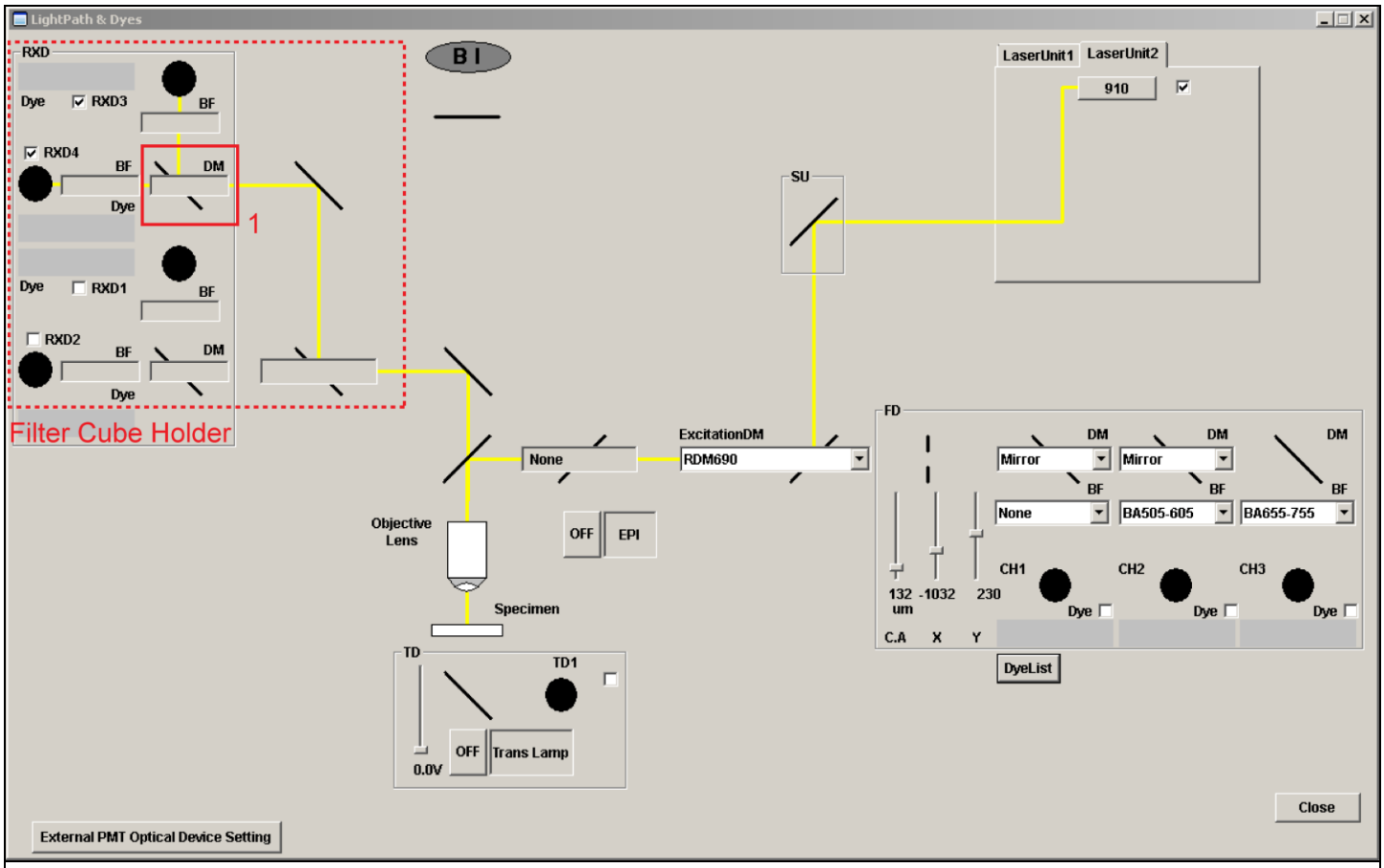
The two images (red boxes 1,2) show simultaneous acquisition of fluorescence emission at two orthogonal state of polarization as detected at PMT3 and PMT4, for a solution of fluorescein diluted in a mixture of water-glycerol (w/w) at 95%.



Supplementary Figure 5.

Alignment polarization control unit.

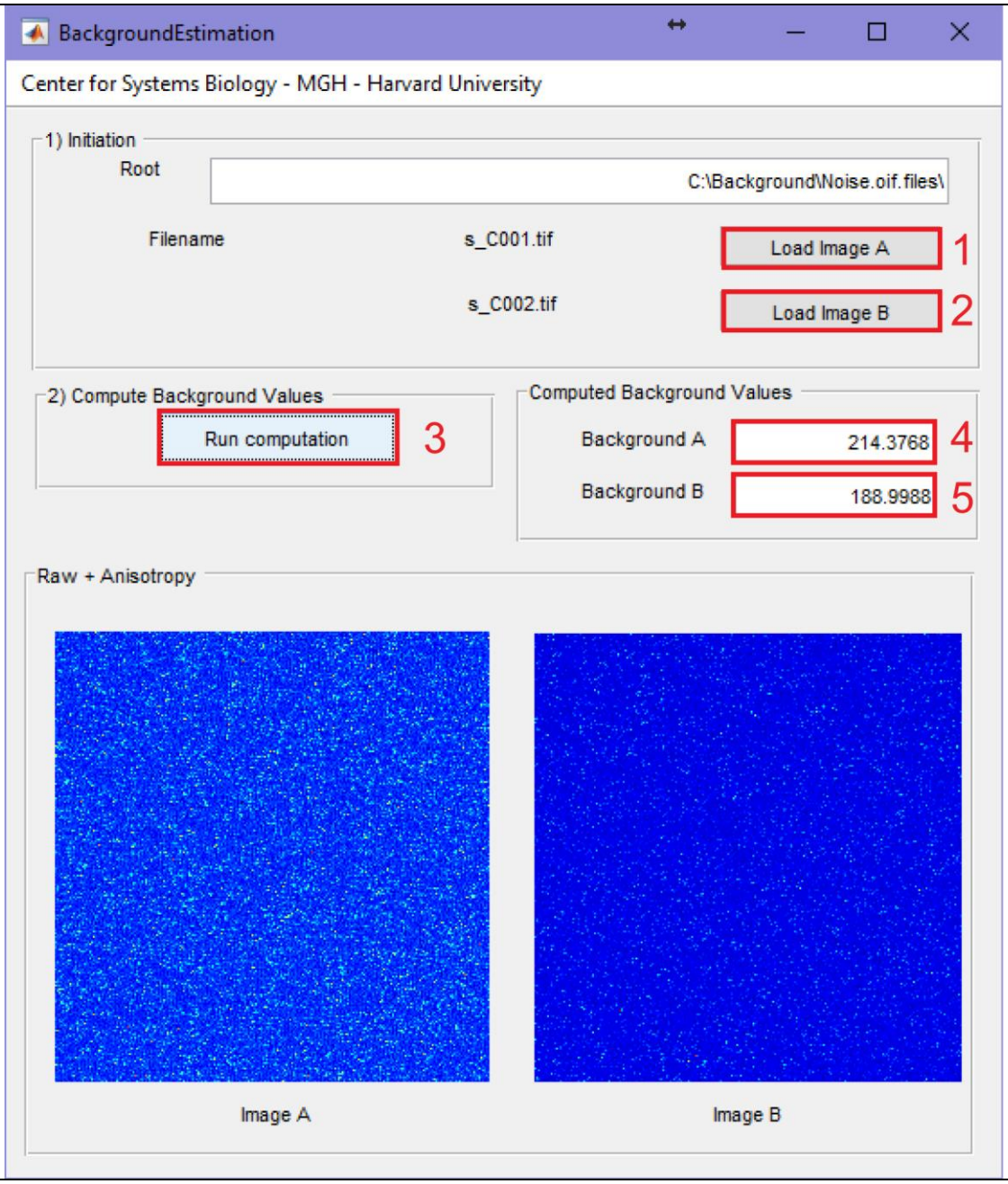
Laser intensity as measured at the backaperture of the objective, as a function of the half-waveplate angle. Light is measured after passing through a near infrared linear polarizer parallel to the microscope Y axis.



Supplementary Figure 6.

LightPath & Dyes window of the Olympus FluoView program.

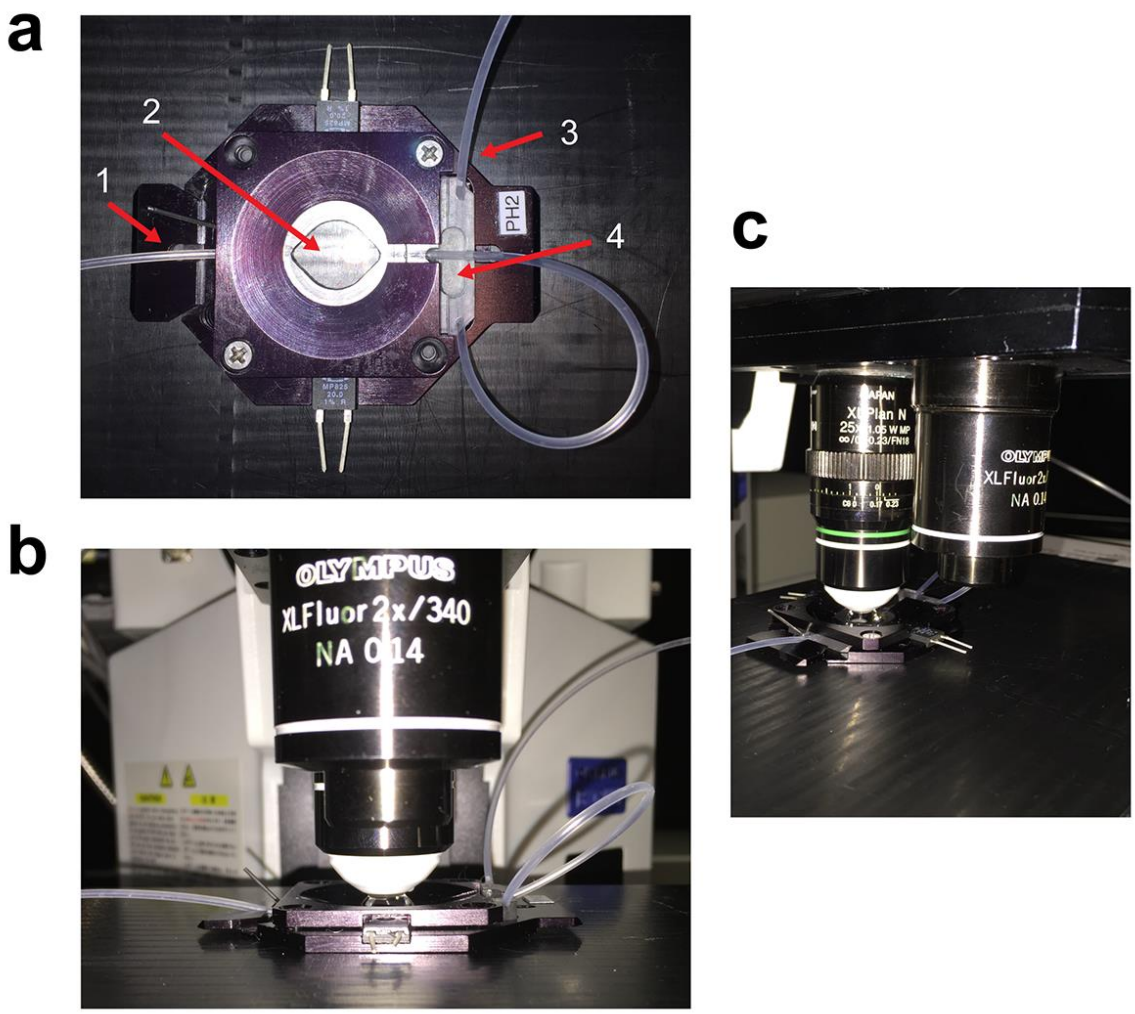
The interface allows to select the dichroic, imaging path, and the imaging PMTs for simultaneous dual channel detection.



Supplementary Figure 7.

GUI interface of the BackgroundEstimation.exe.

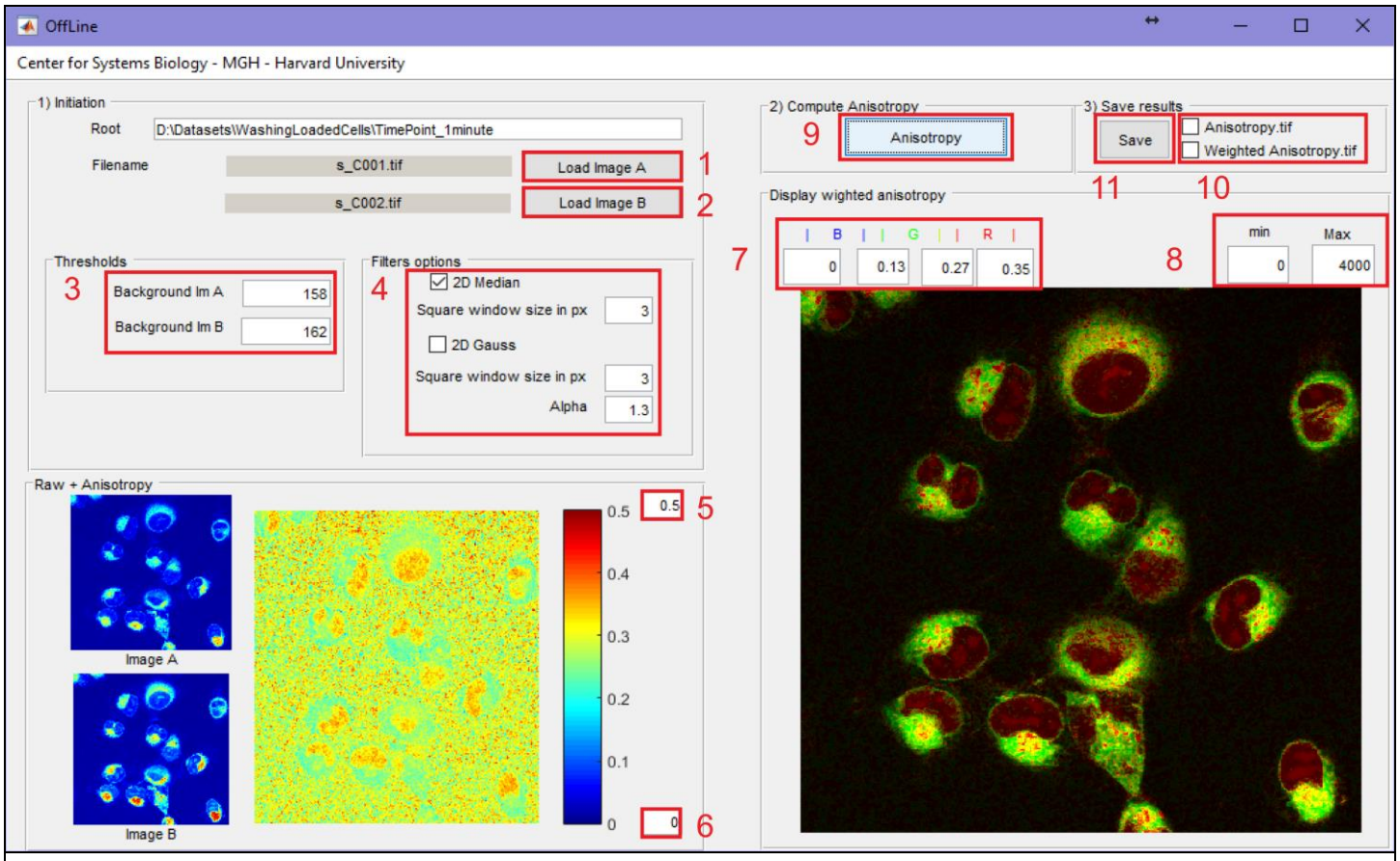
GUI interface of the BackgroundEstimation program, necessary to calculate the dark noise background correction.



Supplementary Figure 8.

Closed bath imaging chamber.

(a-c) Different views of the closed bath imaging chamber for time measurements of drug target engagement. 1, inflow tubing connected the perfusion manifold; 2, cell-seeded coverslip; 3, tubing connected to the vacuum outlet; 4, suction reservoir. The 2x dry objective, is used to find cell-seeded areas on the imaging coverslip.



Supplementary Figure 9.

GUI interface of the OffLine.exe.

GUI interface of the OffLine program, for image processing, calculation and visualization of fluorescence anisotropy images for data previously acquired.