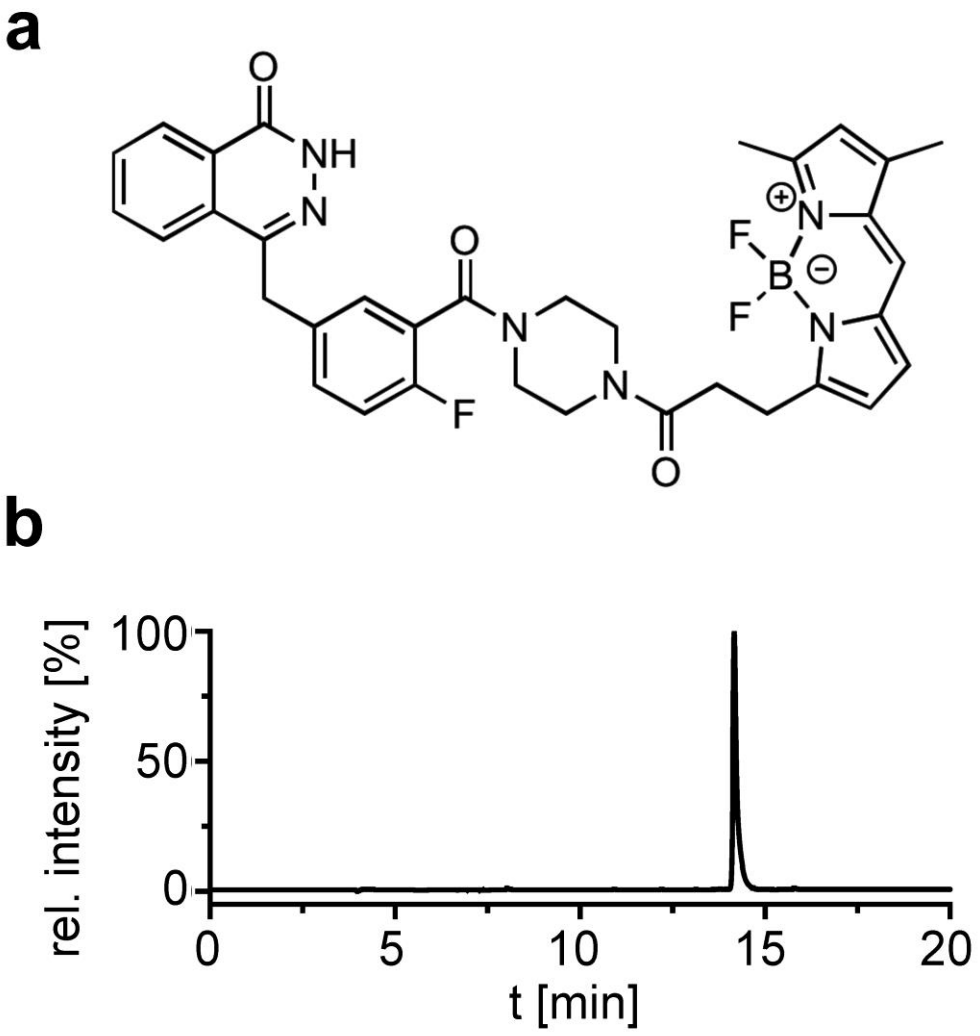
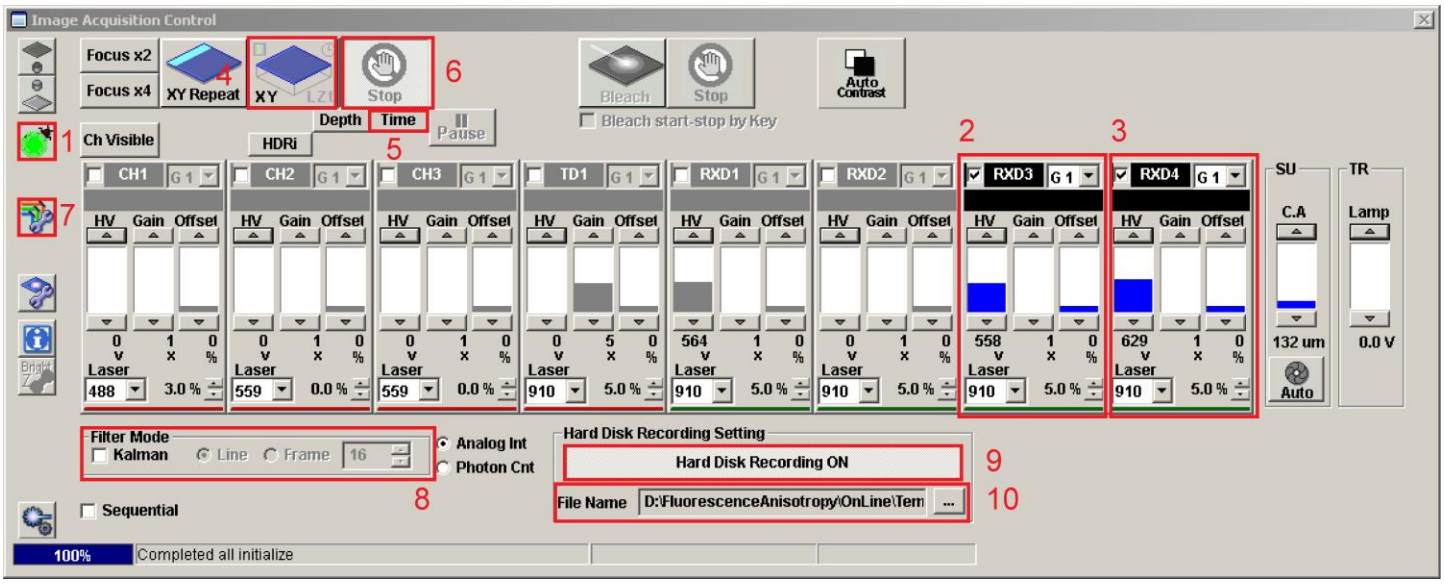


Supplementary Figure 1

PARPi-FL.

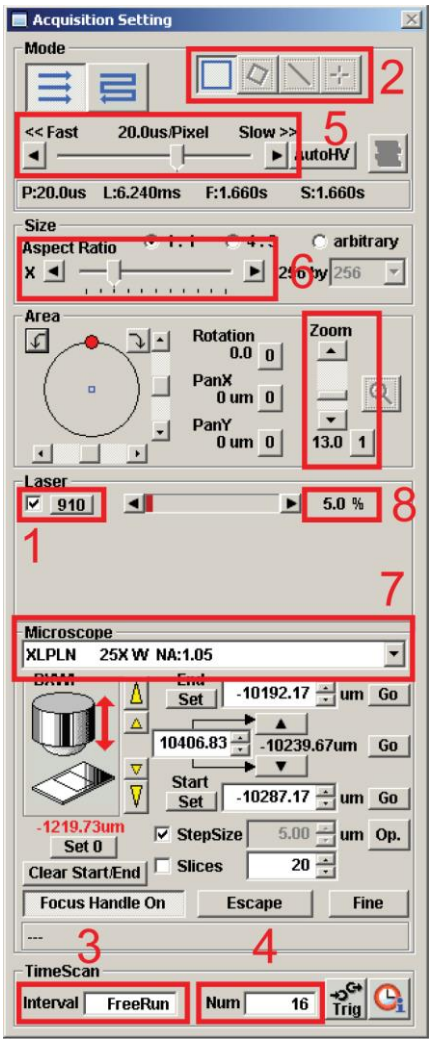
Chemical structure (a) and analytical HPLC chromatogram (b) of PARPi-FL.



Supplementary Figure 2

Image Acquisition Control window of the Olympus FluoView program.

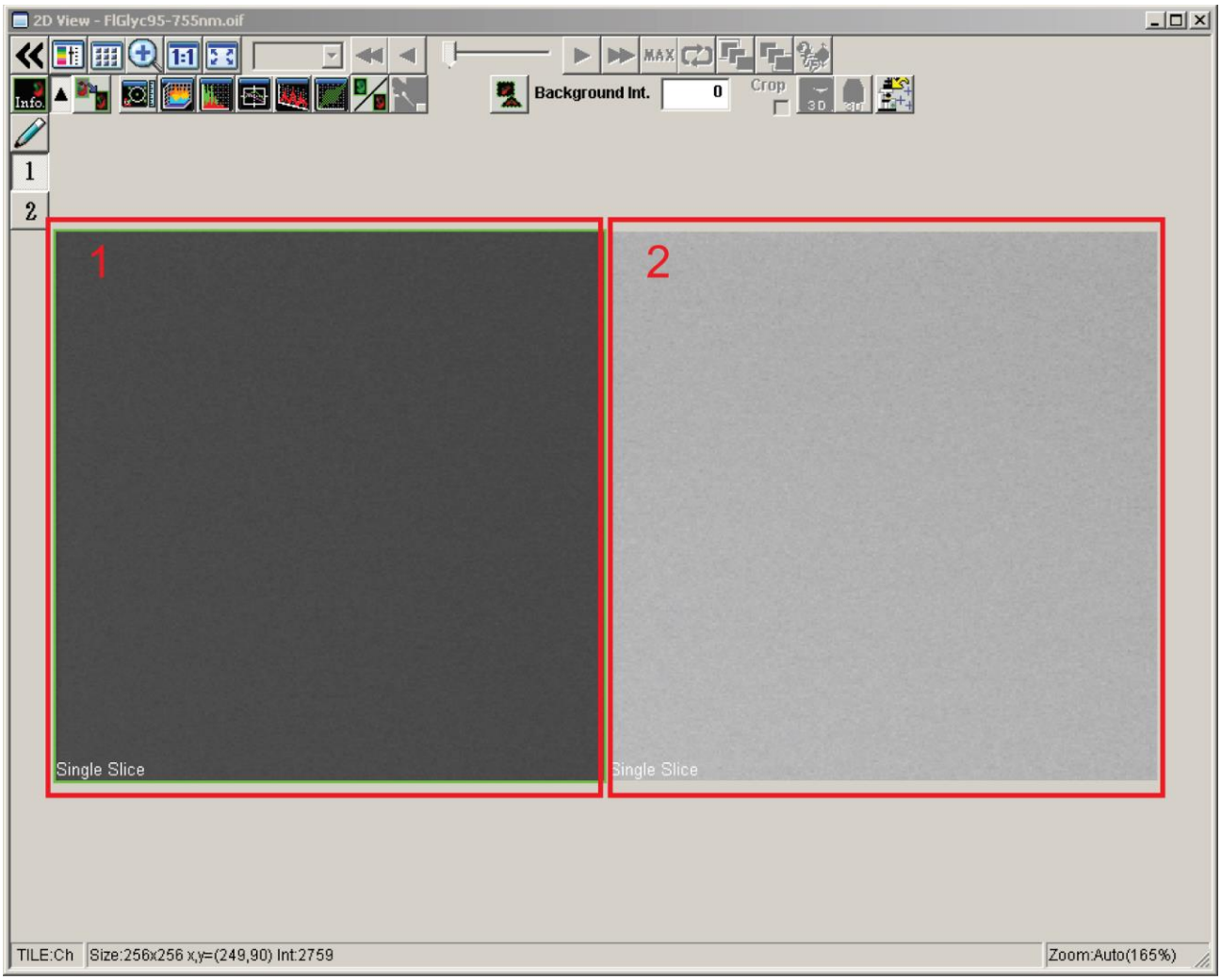
The interface allows to control the PMT voltage settings, to initiate acquisitions and image averaging. Image of FLUOVIEW program adapted with permission from Olympus.



Supplementary Figure 3

Acquisition Setting window of the Olympus FluoView program.

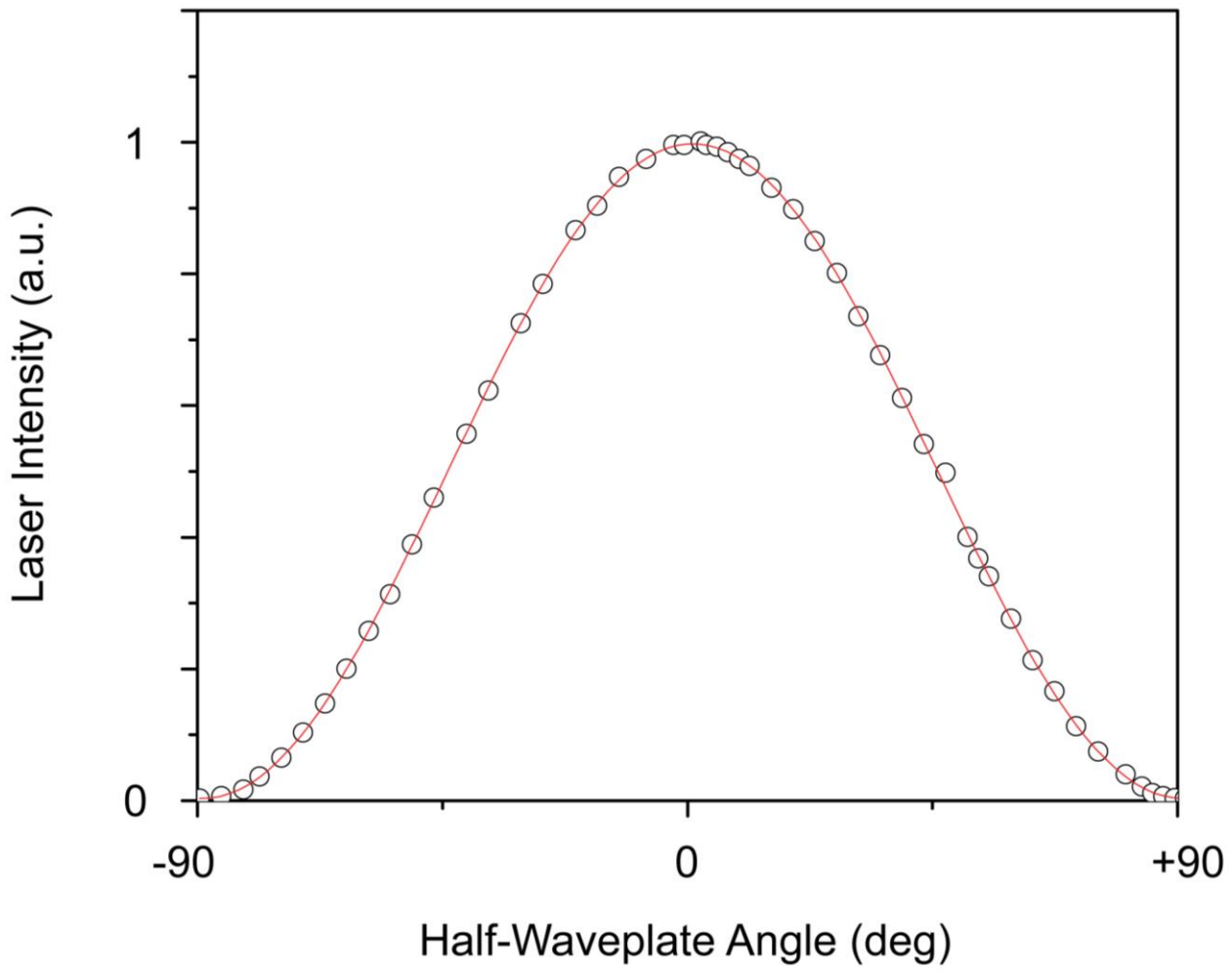
The interface allows to set image acquisition dwell time, size, zoom factor, excitation wavelength selection and laser power. Image of FLUOVIEW program adapted with permission from Olympus.



Supplementary Figure 4

Acquired Image window of the Olympus FluoView program.

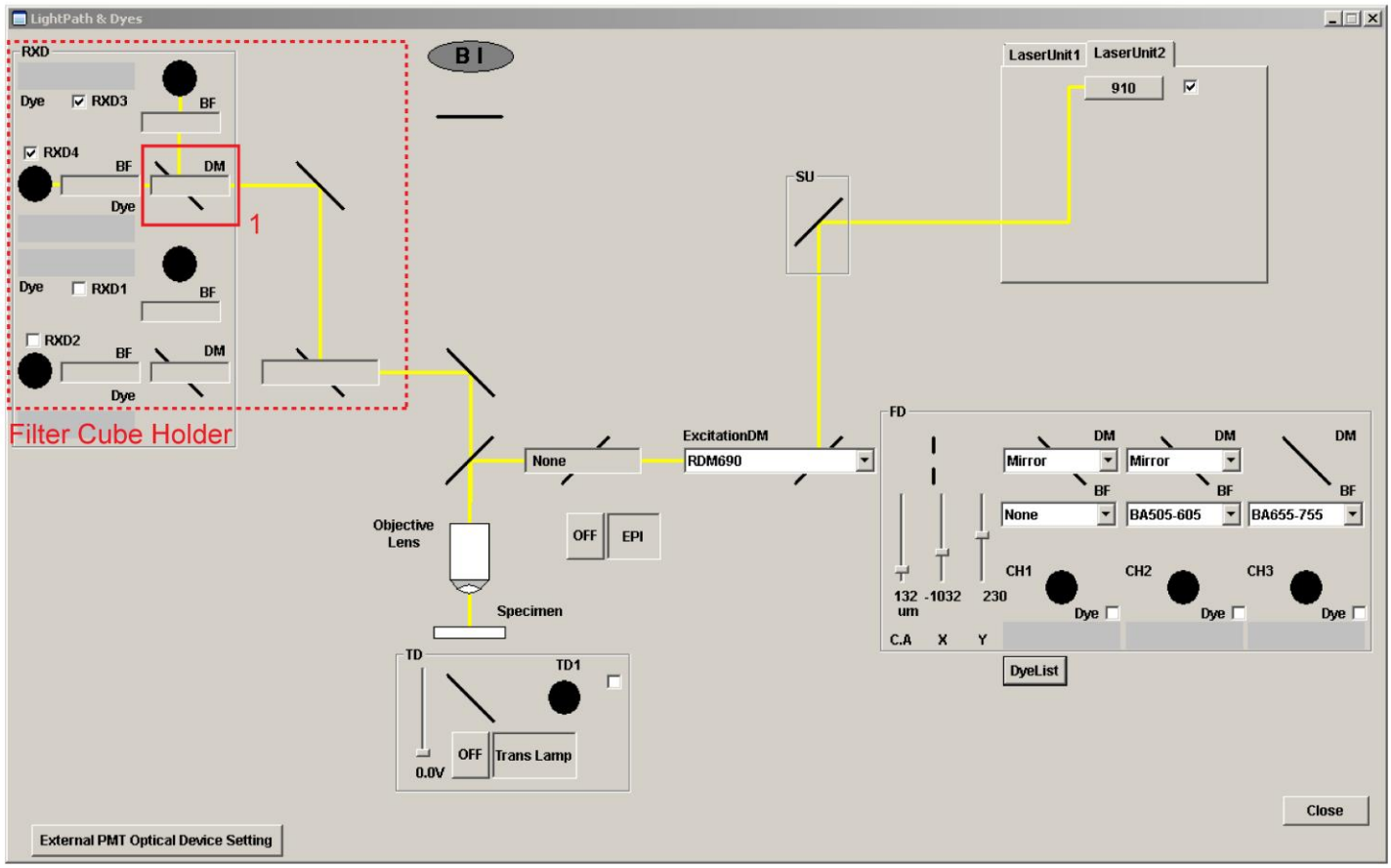
The two images (red boxes 1,2) show simultaneous acquisition of fluorescence emission at two orthogonal state of polarization as detected at PMT3 and PMT4, for a solution of fluorescein diluted in a mixture of water-glycerol (w/w) at 95%. Image of FLUOVIEW program adapted with permission from Olympus.



Supplementary Figure 5

Alignment polarization control unit.

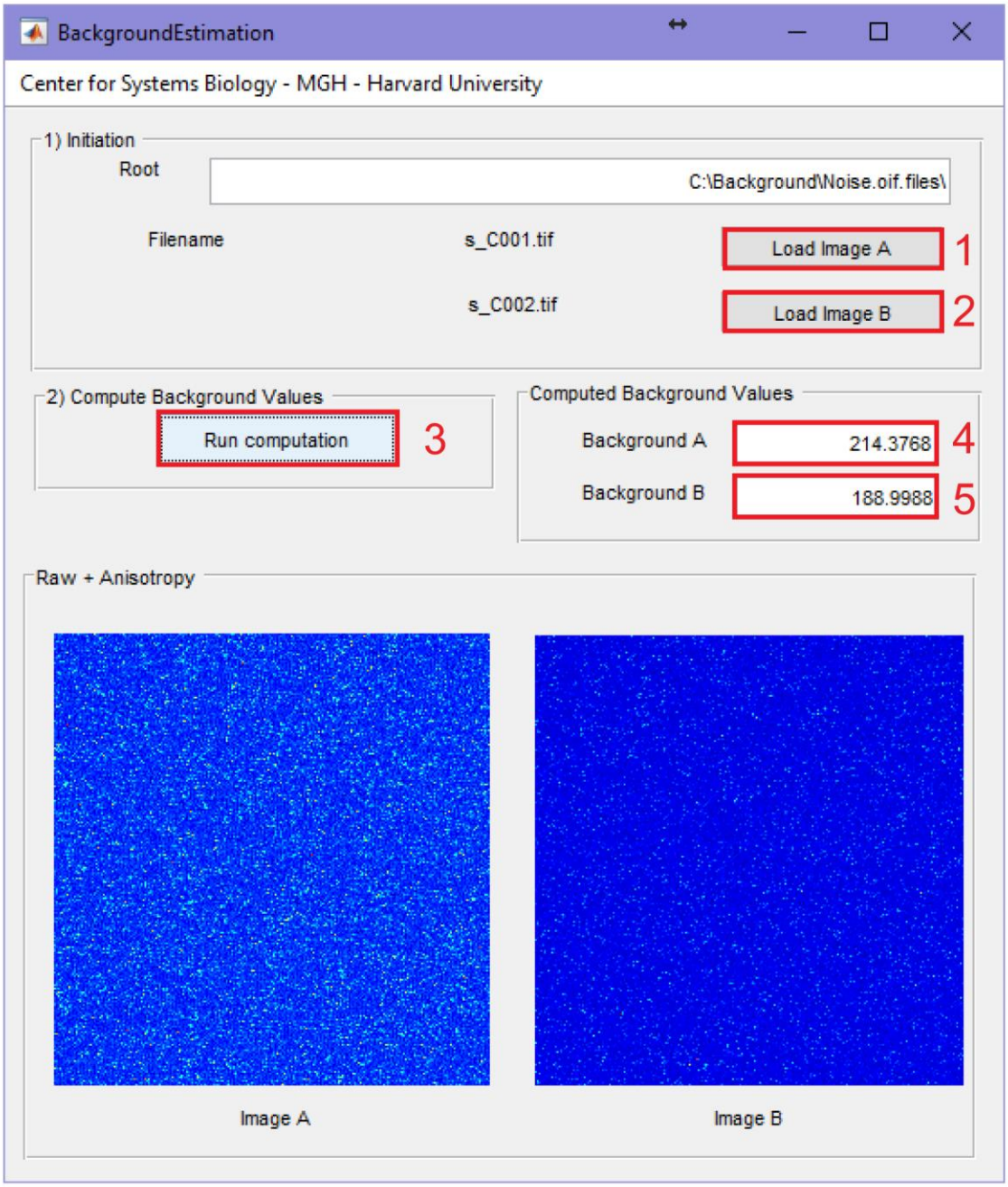
Laser intensity as measured at the back-aperture of the objective, as a function of the half-waveplate angle. Light is measured after passing through a near infrared linear polarizer parallel to the microscope's Y axis (Fig. 4a).



Supplementary Figure 6

LightPath & Dyes window of the Olympus FluoView program.

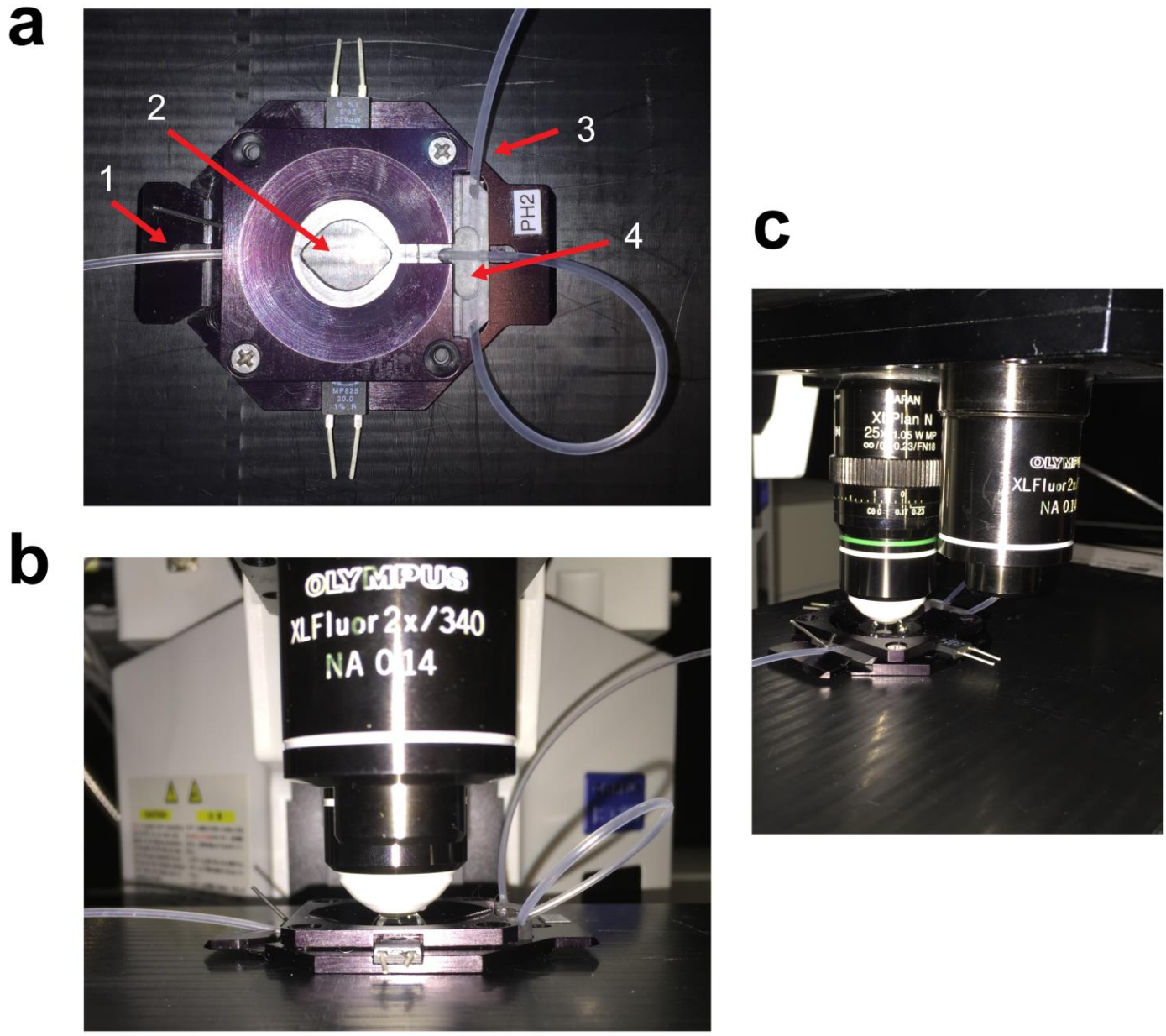
The interface allows to select the dichroic, imaging path, and the imaging PMTs for simultaneous dual channel detection. Image of FLUOVIEW program adapted with permission from Olympus.



Supplementary Figure 7

GUI interface of the BackgroundEstimation.exe.

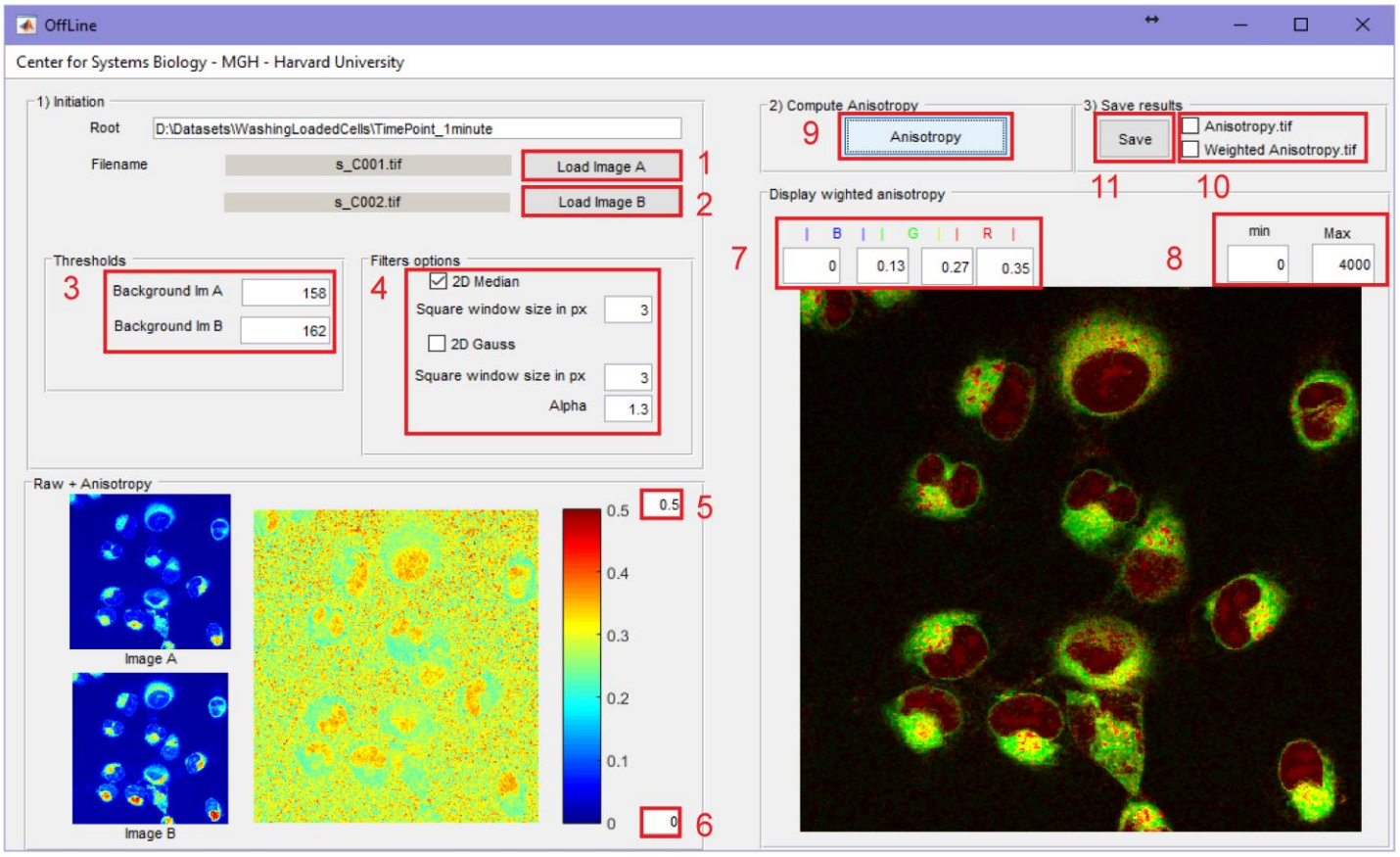
GUI interface of the BackgroundEstimation program, necessary to calculate the dark noise background correction.



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 to 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.

SUPPLEMENTARY INFORMATION:

Measurement of drug target engagement in live cells by two-photon fluorescence anisotropy imaging.

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SUPPLEMENTARY DATA

Supplementary Data 1. One zip file (*SupplementaryData1.zip*) containing the STL file for the assembly of the polarization control unit.
Supplementary Data 2. One zip file (*SupplementaryData2.zip*) containing the STL files for the 3D printing and drawing of the polarization filter cube PFC.
Supplementary Data 3. One zip file (*SupplementaryData3*.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 SOFTWARE

Supplementary Software 1. One zip file (*SupplementarySoftware1*.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 METHODS

Preparation of PARPi-FL

Reagents

* BODIPY-Fl NHS ester (Thermofisher, Cat. No. D-2184, CAS no. 146616-66-2)
* Triethylamine (Sigma-Aldrich, Cat. No. 471283, CAS no. 121-44-8)
  CAUTION It is corrosive and highly flammable.
* PARP1 precursor (MSKCC Reiner lab)
* Olaparib, LC Laboratories 10 mg (Fisher Scientific, Cat. No. NC0136853)
* Acetonitrile extra dry (Fisher Scientific, Cat. No. AC364311000, CAS no. 75-05-8)
  CAUTION It is flammable and harmful.
* Acetonitrile HPLC grade (Fisher Scientific, Cat. No. A998-4, CAS no. 75-05-8)
  CAUTION It is flammable and harmful.
* Milli-Q water (Siemens PureLab Ultra, 18.2 MΩ cm)
* Trifluoroacetic acid (Sigma-Aldrich, Cat. No. T6508, CAS no. 76-05-1)
  CAUTION It is highly corrosive and harmful. Protective lab coat, gloves, and eye protection is needed.
* Dimethyl sulfoxide-d_6 (Sigma-Aldrich, Cat. No. 151874, CAS no. 2206-27-1)
* Formic acid LC/MS grade (Fisher Scientific, Cat. No. A117-50)
  CAUTION It is flammable and corrosive.
* Liquid nitrogen
  CAUTION Liquid nitrogen can cause burns and asphyxiation.
* Nitrogen compressed gas
* Poly (ethylene glycol) 300 (PEG300, Sigma-Aldrich, Cat. No. 90878)

* Fisherbrand Premium microcentrifuge tubes (Fisher Scientific, Cat. No. 05-408-129)
* Phosphate buffered saline (PBS, Fisher Scientific, Cat. No. 20-012-027)
* Standard protective equipment: nitrile gloves, lab coat

Equipment

* 4 mL Borosilicate amber glass vials (Fisher Scientific, Cat. No. 14-955-331)
* Magnetic stirring bar (Sigma-Aldrich, Cat. No. Z328928)
* Ballons (Sigma-Aldrich, Cat. No. Z154997)
* Magnetic stirrer IKA RCT basic (Fisher Scientific, Cat. No. 14-505-602)
* Transferpipets 100-1000 µL (Fisher Scientific, Cat. No. 03-837-322)
* Transferpipets (Fisher Scientific, Cat. No. 21-375E)
* Transferpipets 20-200 µL (Fisher Scientific, Cat. No. 03-837-321)
* Transferpipets (Fisher Scientific, Cat. No. 02-707-410)
* Mettler Toledo XS 105 (Fisher Scientific, Cat. No. 01-913-892)
* Microspatula (Fisher Scientific, Cat. No. 21-401-25A)
* Shimadzu UFLC system (Shimadzu degasser DGU-20A3, Shimadzu pump LC-20AB, Shimadzu detector SPD-M20A, Shimadzu module CBM-20A, Shimadzu fraction collector FRC-10A)
* Phenomenex Jupiter 5 µm C18 300A 10 × 250 mm (Phenomenex, Cat. No. 00G-4053-E0)
* Waters Atlantis T3 10 µm 100A 4.6 × 250 mm (Waters, Cat. No. 186003748)
* Kimax media storage bottles (1000 mL, Fisher Scientific, Cat. No. 02-542D)
* Disposable borosilicate glass tubes (Fisher-Scientific, Cat. No. 14-961-27)
* Corning centrifuge tubes (Fisher Scientific, Cat. No. 05-538-67)
* Parafilm M sealing film (Sigma-Aldrich)
* Hamilton Microliter 700 syringe (500 µL, Sigma-Aldrich, Cat. No. 26222-U)
* Lyophilizer LabConco FreeZone 2.5 Plus (Fisher Scientific, Cat. No. 10-030-139, see Equipment Setup Section)
* Lyophilizer flask (Labconco)
* NMR spectrometer (Bruker Ultrashield 600 Plus)
* NMR sample tubes (Wilmad LabGlass, Cat. No. 535-PP-7)
* Liquid chromatography mass spectrometer (Shimadzu LCMS-2020, see Equipment Setup Section)
* Waters Atlantis T3 5 µm 100A 4.6 × 100 mm (Waters, Cat. No. 186003746)
* Shimadzu Prominence Autosampler Vials (Shimadzu, Cat. No. 220-91521-01)

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, ^3J = 7.0 Hz, 1 H), 2.71 (t, ^3J = 7.5 Hz, 1 H), 2.46 (d, ^3J = 9.3 Hz, 3 H), 2.26 (d, ^3J = 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.