

Automated, quantitative Fast FLIM-FRET (qF³): *A step-by-step protocol to measure dissociation constants for protein-protein interactions in live-cell screening applications*

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Method Article

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Abstract

Fluorescence lifetime imaging microscopy (FLIM) can detect Förster resonance energy transfer (FRET) to measure protein-protein interactions in live cells. Traditional FLIM-FRET uses relative intensities to represent protein expression, consequently limiting users to a qualitative comparison of resulting binding curves. In our quantitative Fast FLIM-FRET (qF³) method we use fluorescent protein standards to convert intensity to concentration and then fit binding curves to determine live-cell apparent dissociation constants (K_d). To acquire measurements with high spatial and temporal resolution in live cells we built a multiplexed system for rapid data acquisition. In this way, complete quantitative binding curves can be generated for hundreds of high-resolution regions of interest from 384 samples in less than 24 hours. In addition, we introduce new automated criteria for quality control in screening. We applied this method to screen a large set of BH3 mimetic drugs targeting BCL-2 family protein dimeric interactions in live cells (associated publication in Science Advances). Here we provide a step-by-step protocol for the qF³ method, which can be adapted for use with other proteins of interest on a multitude of other FLIM-FRET platforms, enabling future automated analysis of protein-protein interactions in live cells at even faster speeds.

Introduction

Protein-protein interactions mediate important processes in the cell and many have been validated as viable but difficult therapeutic targets. Popular methods to measure protein-protein interactions to screen compounds include surface plasmon resonance, nuclear magnetic resonance spectroscopy, and isothermal calorimetry. However, these methods require protein purification for study *in vitro*^{1,2}. Purification of difficult proteins is often achieved by protein truncation, i.e. the membrane-binding region (MBR) is frequently removed to enable measurement of protein-protein interactions³. Truncated or not, purified proteins may not always function the same as inside a cell. Furthermore, the regulatory and compensatory (feedback) pathways regulating protein-protein interactions are missing when purified proteins are used. We recently used fluorescence lifetime imaging microscopy-Förster resonance energy transfer (FLIM-FRET) to measure full-length BCL-2 family protein-protein interactions and test BH3 mimetics live cells⁴. While informative, data acquisition was low-throughput and binding curves were non-quantitative.

In traditional FLIM-FRET methods, two proteins (A and B) are expressed in cells with a fluorophore tag at the N- or C-terminus. Within short distances (<10nm) and with dipole-dipole alignment, the excited “donor” fluorophore can transfer energy to the “acceptor” fluorophore by FRET. The characteristic *fluorescence lifetime* of the donor decreases with FRET in a manner independent of intensity. Thus, if fusion proteins A and B interact in live cells, and bring the donor and acceptor in close proximity for FRET, the experimentalist can reliably measure a decrease in donor lifetime. To facilitate building binding curves, the donor-fusion protein is stably expressed in cells, whereas the acceptor-fusion protein is transiently

transfected. This ensures that the full range of expression levels of the acceptor required to generate a binding curve is present in a single sample (typically a single well of a 384 well plate). The process takes 3 days, but qF³ is simple to perform and requires minimal reagents in a 384-well plate format. Cells stably expressing the donor are seeded into a 384-well plate (day 1), transfected and later drug-treated (day 2) then imaged (day 3). A set of fluorescent protein standards are added to every plate and imaged with each experiment. Subcellular regions of interest (ROIs) are automatically selected within cell images from which the average intensity and lifetime are recorded. Finally, a binding curve is plotted by combining the data from thousands of ROIs. For a true protein-protein interaction, % FRET increases in a hyperbolic manner until it reaches a plateau, indicative of donor saturation, which can then be fit to a Hill slope. In contrast, non-specific interactions such as collisions have a comparably lower FRET that usually increases linearly with the concentration of acceptor. At high local concentrations of the acceptor, such as when two proteins are confined to the same subcellular membrane, random collisions can result in FRET efficiencies that are comparable to that of a protein-protein interaction. Thus, the best practice is to define “binding vs. no binding” by including a mutant or negative control with similar subcellular localization. Intensities of the fluorescence proteins attached to the target fluctuate with changes in laser power or alignment from day to day, contributing to increased error when combining biological replicates. Therefore estimates of the acceptor:donor ratio based on intensities are not comparable for binding curves acquired using different settings or on different microscopes. Additionally, most current FLIM-FRET microscopes are slow and unsuitable for screening.

Our quantitative Fast FLIM-FRET (qF³) method was designed for high content FLIM-FRET data acquisition and analysis (see Osterlund *et al.*, 2022, “Efficacy and specificity of inhibitors of BCL-2 family protein interactions assessed by affinity measurements in live cells”, Science Advances). We built a multiplexed microscope and modified the FLIM-FRET acquisition and the analysis protocol to extract quantitative binding curves. A major change in data acquisition is that within each experiment we image a set of purified fluorescent standards: purified donor (mCerulean3) and acceptor (Venus) protein, each prepared as a standard curve of known concentrations. This data is used to estimate the concentration of donor and acceptor-fusion proteins expressed in live cells within selected ROIs. At every step of the data collection and interpretation process, our analysis has been optimized for screening purposes. Thus, ROIs are automatically selected before determining the donor lifetime, making analysis more accurate and faster. Combined with lifetime data, we calculate the fraction of bound and free donor and acceptor molecules in each ROI. The only assumption required for the analysis is that binding of the proteins of interest occurs at a 1:1 ratio. While this assumption has its limitations, the resulting data can be directly compared and remain informative, and can always be changed to fit a particular experiment. From ROI data, 3D FLIM-FRET binding curves are generated enabling the determination of apparent K_ds for relevant concentration ranges and the identification of concentration ranges in which the apparent K_d is altered by the expression level of the donor-fusion protein in cells. This can be an issue if the concentration of the donor is greater than the K_d as it will change the value of the apparent K_d in cells. To minimize this effect

we analyze data for a restricted range of donor concentration that is biologically relevant (1-3 μ M) and compress curves to 2D. Our analysis is broken down into discrete steps enabling the results to be viewed at any point and allowing users to change the ranges of data used for analysis along the pipeline. Each binding curve is composed of thousands of subcellular ROIs, which are then binned by free acceptor ($Venus_{free}$) concentration. This equalizes the contribution of replicate data when three or more biological replicates are combined. We also introduce two criteria to automate deciding whether screening is possible. First, we define a minimal difference between positive and negative controls that provides sufficient dynamic range to quantify drug-induced changes. Second, we extract information for every curve including the number of points, the range of acceptor and donor protein expression, the K_d , and (for wells treated with inhibitors) the % Resistance⁴, etc. By measuring changes in affinity in live cells, we screened 15 of the top BH3 mimetics available, for disruption of 24 unique BCL-2 family protein-protein interactions (see Osterlund *et al.*, 2022, “Efficacy and specificity of inhibitors of BCL-2 family protein interactions assessed by affinity measurements in live cells”, Science Advances).

Some of the limitations for traditional FLIM-FRET persist: proteins must be overexpressed in cells; protein function/binding may be impacted by the fluorophore tag; proteins with a short half-life may be difficult to express in cells; some protein-protein interactions may not position the donor and acceptor within a favourable position/orientation for FRET to occur. Thus the lack of observed FRET does not always translate to “no binding” (false negative). Our qF^3 process enables automated detection of protein pairs for which FRET detection or quantification is problematic thereby highlighting in a screen those interactions requiring manual interrogation. If desired, problematic protein pairs can be optimized as we demonstrated for the ^VNOXA and ^CBCL-W pair in our associated paper. A limitation of any protein binding experiment in which one is calculating a K_d is that if the protein concentration of the donor exceeds the K_d it will change the apparent K_d calculated (see associated text for further discussion). This is problematic in live-cell experiments where absolute protein concentrations at specific subcellular locations are difficult to control. Nevertheless, when sufficient binding is detected for two proteins of interest, qF^3 is an accurate method for screening both genetic changes and small molecule protein-protein interaction inhibitors in live cells. The speed of qF^3 data acquisition on our multiplexed platform greatly exceeds that of traditional systems as a full 384 well sample plate can be acquired and analyzed in a single day. The automated analyses methods used to facilitate screening by qF^3 can be adapted to any commercial TCSPC FLIM microscope. The benefits of qF^3 include: full-length protein interactions may be studied in their native environment; confocal images provide high-resolution biological information; false positives are a rare event given the requirements for FRET and that collisional controls included in our protocol; previously unknown features of binding and compound function may be revealed in live cells⁴⁻⁵; It is possible to examine protein-protein interactions over time (not shown here); binding curves are quantitative; the supervised, automated analysis is optimized for speed, accuracy, and extraction of maximum information from binding curves.

Here we provide a step-by-step protocol, as well as instructional videos. This is meant to complement our upcoming associated publication and application of the qF³ method (see Osterlund *et al.*, 2022, “Efficacy and specificity of inhibitors of BCL-2 family protein interactions assessed by affinity measurements in live cells”, Science Advances).

Reagents

General plastics

- 15mL falcon tubes (FroggaBio TB15-500)
- 50mL falcon tubes (FroggaBio TB50-500)
- 1.7mL tubes (FroggaBio LMCT1.7B)
- Tissue culture plastics:
 - 10cm dish (Starstedt Ref 83.3902)
 - 6 well plate (Starstedt Ref 83.3920)
 - CellCarrier-384 Ultra microplate, tissue culture treated, black 384-well with lid (PerkinElmer, 6057300)
 - 1.7mL tubes, autoclaved

DNA preps

- NEB® 10-beta competent E. coli (High Efficiency) (New England Biolabs, C3019H), expanded in house, made chemically competent, and used for DNA preparation
- GeneJet Plasmid Midiprep kit (ThermoFisher Scientific, Cat. No. K0481)
- GeneJet Plasmid MiniPrep kit (ThermoFisher Scientific, Cat. No. K0503)

Fluorescent protein purification

- His6-TEV-Venus-pBluescript (Addgene ID # 166756)

- His6-TEV-mCerulean3-pBluescript (Addgene ID # 166757)
- BL21-AI™ One Shot™ Chemically Competent E. coli (Thermo FisherScientific, Cat. No. C607003) expanded in house, made electrocompetent, and used for growing protein
- Luria Burtani (LB) broth: To 1L MilliQ H₂O add 10g NaCl + 10g Tryptone (Bioshop TRP402.5) + 5g Yeast extract (Bioshop YEX401.205), then autoclave.
- LB-Agar: to 250mL LB broth add 3.75 grams Agar (Bioshop AGR003.1), then autoclave.
- Ampicillin (Bioshop, Cat. No. 69-52-3)
- 250mL glass erlenmeyer flask
- 2.8 L Pyrex® Fernbach culture flask (Sigma CLS44202XL)
- 1L centrifuge bottle polycarbonate (Beckman Coulter 366751)
- L-(+)-Arabinose (Bioshop, Cat No. ARB222.500)
- Dnase I from bovine pancreas (Sigma DN25)
- 200x PIN (protease inhibitor cocktail): add 20μL chymostatin(10mg/mL in DMSO), 20μL Antipain (10mg/mL in DMSO), 20μL leupeptin (10mg/mL in H₂O), 20μL pepstatin (10mg/mL in H₂O), 200μL aprotinin (2mg/mL in H₂O) and 9.72 mL water to total 10 mL volume. Mix and freeze 500μL aliquots at -80°C.
 - Chymostatin (Sigma-Aldrich, Cat. No C7268)
 - Antipain (Sigma-Aldrich, Cat. No A6191)
 - Leupeptin (Sigma-Aldrich, Cat. No L2884)
 - Pepstatin (Sigma-Aldrich, Cat. No P5318)
 - Aprotinin (Sigma-Aldrich, Cat. No 10820)
- 100x PMSF (Serine protease inhibitor): add 17.5 mg Phenylmethylsulfonyl fluoride (Bioshop, Cat No. PMS123.25) to 1mL 95% ethanol (0.1M= 100x stock).

! Critical: *Prepare immediately before use.* Storage at -20°C and use anhydrous ethanol or isopropanol.*

- Ni-NTA Agarose (Invitrogen, Cat. No. R901-15)
- Imidazole (Bioshop IMD508.1)
- Fisherbrand™ Regenerated Cellulose Dialysis Tubing, MW 12000-14000 Da (FisherScientific 21-152-17)

Cell culture

- Baby Mouse Kidney (BMK) and BMK-DKO (BMK with double gene knockout of Bax and Bak) cells were a gift from Dr. Eileen White (Rutgers University, PMID: 11836241)
- HEK293T cells (*H.sapiens*) were purchased from ATCC (ATCC® CRL-3216™)
- Fetal bovine serum (FBS) (Sigma Cat. No. 16C423)
- Phosphate buffered saline (PBS)_pH 7.2: 10mM Na₂HPO₄, 1.8mM KH₂PO₄, 2.7mM KCl, 137mM NaCl
- VacuCap 90 Filter unit w/ 0.1µm Supor Membrane (Life Sciences 4621)
- αMEM complete media pH 7.2: Serum-free αMEM made from powder as directed (Gibco™ MEMα no nucleosides, Fisher Scientific, Cat. No. 12000022) and filtered through a 0.1µm Supor Membrane. For complete media add 10% FBS and 1% 100x Penicillin-Streptomycin.
- DMEM complete media pH 7.2: Serum-free DMEM made from powder as directed (Gibco™ DMEM high glucose powder, Fisher Scientific, Cat. No. 12100061) and filtered through a 0.1µm Supor Membrane. For complete media add 10% FBS, 1% 100x Penicillin-Streptomycin and 1% 100X MEM Non-Essential Amino Acids.

- Cell freezing media: 45% serum-free media (whichever used for that cell line), 50% FBS, 5% DMSO.
- Gibco™ Trypsin-EDTA (0.5%), no phenol red (FisherScientific, Cat. No. 15-400-054)
- 100X MEM Non-Essential Amino Acids (Gibco, Cat. No. 11140076)
- Penicillin-Streptomycin (FisherScientific, Cat. No. 15-140-122)

Ψ Note: *All cell lines are routinely tested for mycoplasma contamination.*

Transfection reaction

- Midiprep of pSPUTK (Stratagene Santa Clara CA, Cat. No: CB4278654)
- TransIT-X2 Transfection Reagent (Mirus, Cat. No: Mir 6003)
- Midiprep of each plasmid to be included in the screen

Ψ Note: *See Table 2 of the associated manuscript for a complete list of plasmids used in our Bcl-2 family protein inhibitor screen.*

Determining G-factor

- Parental cell line (ie. we used BMK-DKO cells⁵)
- mVenus-pEGFP-C1 (GI: KU341334.1, received as a gift from Dr. Ray Truant (McMaster University))
- mCerulean3-pEGFP-C1 (Addgene ID #54730, received as a gift from Dr. Mark A Rizzo (University of Maryland))
- mCerulean3-5aa-Venus-pEGFP-C1 (Addgene ID # 26394)
- mCerulean3-13aa-Venus-pEGFP-C1 (Addgene ID # 166753)

- mCerulean3-31aa-Venus-pEGFP-C1 (Addgene ID # 166754)
- mCerulean3-39aa-Venus-pEGFP-C1 (Addgene ID # 166755)

Standards for qF³ image collection

- 10nM Fluorescein in 0.1M NaOH. Store 50mL volume in 4°C, covered to prevent light exposure.
- Fluorescein sodium salt (Sigma-Aldrich, Cat. No. F6377-100G)
- Quenched fluorescein: *prepared as we recently outlined*⁶. Stored 50mL volume at room temperature, covered with aluminum.

Ψ Note: *Aggregates may form after preparation. We filtered our quenched fluorescein sample through a 0.2 μM filter and observed consistently homogeneous images for >1 year following.*

- Standard curve of purified mCerulean3 at known concentrations (*we used 0-8μM*)
- Standard curve of purified Venus at known concentrations (*we used 0-50μM*)

Ψ Note: *See later, the procedure for fluorophore purification.*

Equipment

See our associated manuscript (Osterlund *et al.*, 2022, “Efficacy and specificity of inhibitors of BCL-2 family protein interactions assessed by affinity measurements in live cells”, Science Advances) where we have provided a detailed diagram and description of the INO-FLIM Hyperspectral (HS) microscope in the Supplementary text.

Procedure

PREPARATORY EXPERIMENTS

1. Prepare standard curves of fluorophores

Ψ Note: *With every qF^3 experiment, we imaged standard curves for the fluorophores.*

Below we include instructions for the purification of these fluorophores:

A) Protein expression in *E. coli*

- a. Transform BL21-AI™ One Shot™ Chemically Competent *E. coli* with DNA construct, “His₆TEV-mCerulean3-pBluescript” or “His₆TEV-Venus-pBluescript” onto LB-agar.
- b. Select a single colony and grow overnight in 50 mL Luria Broth (LB) + 100µg/mL Ampicillin (AMP) at 37°C, shaking (250rpm).
- c. Prepare two 1.5 litre LB-AMP. Add 25 mL of overnight culture to each then grow at 37°C, shaking (250rpm) until OD₆₀₀ reaches 0.6
- d. Induce with 0.2% L-Arabinose
- e. Reduce temperature to 30°C
- f. Incubate for 3 more hours, shaking.
- g. Harvest bacterial pellet via centrifugation. (4500xg, 15min). Discard the supernatant and freeze pellet (-20°C until purification).

B) Prepare buffers for protein purification

Ψ Note: *For purification chill all buffers to 4°C and adjust final pH to 7.5.*

- a. 100mL Lysis Buffer: 50mM NaH₂PO₄ pH 8.0, 300mM NaCl, 10mM Imidazole, 1%

CHAPs, 2mM MgCl₂.

Ψ Note: *Add fresh: 2x PIN and 1x PMSF and 10µg/mL DNase.*

b. 100mL Wash Buffer: 50mM NaH₂PO₄ pH 8.0, 300mM NaCl, 10mM Imidazole.

c. 20mL Elution Buffer: 50mM NaH₂PO₄ pH 8.0, 300mM NaCl, 250mM Imidazole.

d. 4L Dialysis Buffer: 50mM NaH₂PO₄ pH 7.5, 150mM NaCl.

C) Purify fluorophore protein

Ψ Note: *Collect small samples throughout each step for SDS PAGE.*

a. Resuspend pellet in 5mL Lysis Buffer per gram of pellet.

b. Lyse bacteria (*we use a French press*)

c. Centrifuge (22000xg, 20min, 4°C)

d. Transfer supernatant to new 50mL falcon tube and discard the pellet.

e. Wash 1mL Ni-NTA Agarose slurry in 10mL Lysis buffer then add beads to
the supernatant

f. Incubate on rotation (4°C, 1h)

g. Pour supernatant + Ni-NTA slurry into a 15mL column, the resin will collect at the
bottom. Collect the supernatant and gently pour over the resin two more times.

h. Wash the Ni-NTA resin with 50-100mL Wash buffer (*until the flow-through is colourless*)

i. Prepare 10x 1.7mL Microcentrifuge tubes and label: E1 (elution 1), E2, E3... E10.

j. Add 10mL elution buffer. Let the first 1mL pass, then collect fluorophore in 10x 1mL
fractions.

k. Combine five fractions that have the highest intensity.

l. Dialyze protein, stirring at 4°C: (1L for 4h, 2L overnight, 1L for 4h).

m. Remove from dialysis tubing and centrifuge protein at 21000xg, 10min.

Ψ Note: *If there is any pellet (precipitation) transfer supernatant to a new tube.*

n. Add 20% Glycerol to dialyzed protein. Keep at 4°C, until concentration determined and standard curves prepared (in our experience these fluorophores are stable at 4°C for > 1 year).

D) Determine the concentration

Ψ Note: *Fluorescence of these fluorophores interferes with Bradford and BCA assays. We ran a Coomassie-stained SDS PAGE gel with a known protein, approximately the same size (BCL-XL), to estimate concentration in associated text.*

a. Once concentration is known, make serial dilutions of mCerulean3 and Venus proteins in Dialysis buffer + 20% Glycerol.

b. Store 500μL aliquots at -80°C.

2. Determine G factor (optional depending on the instrument)

Ψ Note: *This step is required to correct for the overestimate in $Venus_{free}$ concentration due to FRET stimulated emission. This step was necessary on our INO-FHS instrument because the acceptor channel detection is not synchronized with acceptor excitation. On a system with time-gated acceptor detection, this step is not necessary as the stimulated emission can be temporally gated out. To determine G Factor we based our experiment on the work done by Butz et al., 2016⁷. Comparatively, our analysis is simplified because FRET efficiency is known from the lifetime. The rationale for the G-factor analysis is included in the Supplementary methods of our recent publication. Before this experiment, we had already determined*

the optimal imaging configuration (filter settings/acquisition time/objective/zoom, etc.) for rapid acquisition of live cell samples⁶. The following is the method used to determine the G-factor:

A) Prepare sample (3 days)

- a. Seed 20 wells of a CellCarrier-384 Ultra microplate well plate with 2000 BMK-DKO cells per well in a total volume of 25 μ L DMEM complete media.
- b. Leave plate at room temperature for 15 mins to allow cells to settle before returning to the incubator (37°C, 5% CO₂) for 12-24h
- c. Transfect 2 wells each with plasmids encoding:
 - i) nothing
 - ii) mCerulean3
 - iii) Venus
 - iv) mCerulean3 + Venus
 - v) mCerulean3-5aa-Venus
 - vi) mCerulean3-13aa-Venus
 - vii) mCerulean3-31aa-Venus
 - viii) mCerulean3-39aa-Venus

Ψ Note: *We used TransIT-X2 Transfection reagent and transfected cells as detailed in Osterlund et al., 2019⁶. We add pSPUTK plasmid (Stratagene Santa Clara CA, Cat. No: CB4278654) to every transfection reaction to minimize overexpression while maintaining a constant amount of DNA in all of the transfection reactions.*

All transfection reactions are prepared as follows:

Ψ Note: *Reaction for transfection of 2 wells.*

1. In a 1.7mL microcentrifuge tube add 50ng DNA of interest, 50ng pSPUTK, 9ul Opti-MEM and 0.4μL Mirus reagent
2. Vortex transfection reaction
3. Centrifuge transfection reaction briefly (burst)
4. Incubate 15-30 mins (maximum) at room temperature.

! CRITICAL: do not leave standing more than 30 min

5. Add 50ul of complete media and mix gently by pipetting
6. Add 25ul of this transfection reaction on top of seeded cells.
7. Return plate to the incubator for 3-5h
8. Remove media with a pipette and add fresh complete media

Ψ Note: Changing media minimizes toxicity caused by the transfection reagent.

Ψ Note: Alternatively, treat cells at this time point (**Section 7.B**).

- d. Incubate plate 12-24h (37°C, 5% CO₂)

B) Collect FLIM-FRET data on INO-FHS (2h)

- a. Add the following standards to empty wells in the sample plate:

- i) Venus protein standard curve
- ii) mCerulean3 protein standard curve
- iii) Quenched fluorescein
- iv) 10nM fluorescein in 0.1M NaOH

b. Load imaging configuration (excitation wavelengths/filter settings) matching selected settings that will be used for screening.

Ψ Note: *Detailed method for selection of image acquisition settings and operation of our INO-FHS instrument is included elsewhere⁶.*

c. Acquire several images per well (*we acquired ten 200μm by 200μm images per sample*) with each of three excitation configurations:

- i) Acceptor excitation only (510nm)
- ii) Donor and acceptor excitation together (510nm + 433nm)
- iii) Donor excitation only (433nm)

C) Analyze data to determine G-factor

Ψ Note: *Analyze protein standard curve using the method described later in Analysis Section 2: The analysis will yield two values, the slopes of the mCerulean3 and Venus standard curves (mc3_slope and ven_slope).*

a. For the cell samples, run the region of interest (ROI) segmentation analysis using INO_Analysis software as described later in Analysis Section 3. This

generates CSV files, which contain FLIM and spectral profiles for each ROI.

Ψ Note: *for fitting analysis in the INO-FHS analysis software set 'A0' to 100 and click the checkbox next to A0. This fixes the fitting analysis to a single exponential because the lifetime of mCerulean3-Venus dimer constructs is homogenous.*

b. Copy the generated .CSVs into a single folder (CSV directory) and note the Well IDs used (ie. A1, A2 etc).

c. Download MATLAB scripts from GitHubLink:

https://github.com/DWALab/Osterlund_et_al_2021.git

subfolder: Gfactor_Estimation_Code

i) GFactorByMultipleCSVs.m

ii) CalcConcentrationsV2.m

d. Open script 'GFactorByMultipleCSVs.m'

e. Copy the path to CSV directory

f. input the data_path parameter with the path to CSV directory

g. input mc3_slope

h. input venus_slope

i. input the Well ID for sample 1c: ii. mCerulean3 (mc3_only_well_id)

j. Similarly, input the Well IDs for remaining dimer constructs.

Ψ Note: *In our case, we have 4 constructs/samples with varying amino acid lengths (**section 2.A.c:** v/vi/vii/viii), which are inputted as "construct_1_well_id", "construct_2_well_id", "construct_3_well_id", "construct_4_well_id".*

k. Run GFactorByMultipleCSVs.m script.

Ψ Note: *The script will first open the mCerulean3 only wells to determine bleedthrough into the acceptor channel and the lifetime of mCerulean3 expressed in cells. To reduce noise effects on estimated lifetime only analyze ROI profiles that have, on average, mCerulean3 concentrations equal to and above 0.5 μ M and lower than 5 μ M. Optionally, this range can be modified based on user preference. The script will generate a 3D plot for FRET vs total mCerulean3 concentration and total Venus concentration. This profile is fit in 3D to estimate the G-factor.*

l. The G factor will be displayed. Copy G factor to notes (use later).

3. Generate stable cell lines expressing donor (mCerulean3) fused to a protein of interest

Ψ Note: *We used baby mouse kidney (BMK) cells with BAX and BAK genes knocked out (DKO), to remove complications in screen analysis due to induced cell death with BH3 protein expression or BH3 mimetic treatment. Cells were routinely tested for mycoplasma. The mCerulean3 fusion protein is referred to as the donor.*

A) Clone gene of interest as a fusion with mCerulean3, joined by a flexible linker

Plasmids encoding N-terminal fusions of mCerulean3 to four anti-apoptotic proteins:

a. mCerulean3-BCL-XL-s2193

b. mCerulean3-BCL-2-s2193

c. mCerulean3-BCL-W-FUGW

d. mCerulean3-MCL-1-pLVX

Ψ Note: Throughout this text, we use superscript “C” to indicate N-terminal fusion of mCerulean3. ie. mCerulean3-MCL-1 is shortened to ^CMCL-1.

Ψ Note: We chose N-terminal protein fusion since it did not disrupt protein localization or function⁷. Alternatively, C-terminal fluorescent protein fusions are possible as long as protein function is not affected, and so long as sufficient FRET can be detected with acceptor-fused interacting proteins.

B) Depending on the type of plasmid, transfect or infect cells with the construct of interest

Ψ Note: For genes in s2193 backbone we transfected BMK-DKO cells, selected with 5 µg/mL Blasticidin for two weeks then sorted for mCerulean3 expression. For genes in FUGW and pLVX backbone, we transfected HEK-293T cells with viral packaging components (PAX2 and VSVG), harvested virus after 48-72h, and infected BMK-DKO cells. For selection, 4ug/mL Puromycin was used to generate the BMK-DKO-^CMCL-1 cell line. No antibiotics were necessary for creating our BMK-DKO-^CBCL-W cell line as viral titre was high enough such that selection/sorting was unnecessary.

C) Select and/or sort for mCerulean3 expression in cells

D) Culture cells for 1-2 weeks.

Ψ Note: We repeated sorting until ^Canti-apoptotic protein is expressed in >90% of cells.

E) Expand and freeze early passages of the stable cell line

F) Test fusion-protein function and stability

Ψ Note: *We tested the anti-apoptotic function of our mCerulean3-fusion proteins in another cell line, MCF7 breast cancer cells because we need BAX and BAK proteins present for cells to die. We assume the function is maintained in BMK-DKO cells.*

? Troubleshoot: *If fusion protein is not functional try changing the location of mCerulean3 fusion to the C-terminus. Alternatively, try increasing linker length between the protein of interest and the fluorophore.*

4. Assemble constructs to transiently express acceptor (Venus) fused to a protein of interest

Ψ Note: *If structure N-terminal region of protein of interest has a critical function, then design C-terminal fusion instead, adding a stop codon at the end of mCerulean3.*

A) Create a plasmid encoding an acceptor fusion protein by inserting the cDNA for the gene of interest downstream of Venus in pEGFP-C1 vector. Include a flexible linker between the Venus and target protein. *For example, we generated constructs encoding Venus fused to the N-terminus of each of six BH3-only(^VBH3) proteins:*

a. Venus-BAD-pEGFP-C1

b. Venus-BIML-pEGFP-C1

c. Venus-tBID-pEGFP-C1

- d. Venus-PUMA-pEGFP-C1
- e. Venus-BIK-pEGFP-C1
- f. Venus-NOXA-pEGFP-C1

B) Assemble plasmids encoding collisional control proteins; ie the protein of interest with mutation(s) to eliminate binding. *For example, for each ^VBH3 protein, we made four mutations of critical residues in the BH3 binding regions to Glutamic acid (4E mutation):*

- a. Venus-BAD-4E-pEGFP-C1
- b. Venus-BIML-4E -pEGFP-C1
- c. Venus-tBID-4E -pEGFP-C1
- d. Venus-PUMA-4E -pEGFP-C1
- e. Venus-BIK-4E -pEGFP-C1
- f. Venus-NOXA-4E -pEGFP-C1

! Critical: *The collisional control is required for screening analysis later. This negative control is necessary to distinguish true binding from the increased likeliness that two proteins will collide when colocalized on a membrane surface. If the binding site is unknown a non-binding Venus-fused protein of interest of approximately the correct size and localization can be used. We have made some constructs available for this purpose:*

- a. Venus-7aa-ActA-pEGFP-C1 (Addgene ID # 177405), mitochondrial localization, facing cytoplasm
- b. Venus-7aa-MAO-pEGFP-C3 (Addgene ID # 166765), mitochondrial localization, facing cytoplasm
- c. Venus-7aa-Cb5-pEGFP-C1 (Addgene ID # 166764), endoplasmic reticulum

localization facing cytoplasm

5. Prepare 1000x stocks of compounds of interest

We used DMSO as a solvent to dissolve BH3 mimetic compounds to be tested in a qF³ screen. Whatever compound/solvent is used we suggest keeping dilution consistent throughout the plate. BH3 mimetics were frozen/thawed no more than twice.

A) Dissolve BH3 mimetic in fresh anhydrous DMSO to make 20mM stocks.

B) Store 5µL aliquots at -80°C, desiccated, for future use.

QUANTITATIVE FAST FLIM-FRET (qF³) SAMPLE PREPARATION & DATA ACQUISITION

Ψ Note: *In our associated paper, mCerulean3 fused to an anti-apoptotic protein of interest (^Canti-apoptotic) was stably expressed in BMK-DKO cells (BMK-DKO-^Canti-apoptotic). Stable cells were transfected with Venus-fused to a BH3 protein of interest (^VBH3) to measure the binding curve for ^Canti-apoptotic + ^VBH3. We treated this interaction with a panel of BH3 mimetic drugs, at several concentrations to measure the efficacy of these compounds to inhibit the interaction between ^Canti-apoptotic & ^VBH3. These terms are used in the example below.*

6. Plan screen platemap to include the following:

A) One column of empty wells

Purpose: space for fluorescence protein standards

B) Untransfected BMK-DKO-^Canti-apoptotic cells treated with DMSO diluted 1000x in complete media.

Purpose: To determine the lifetime of the donor alone, ^Canti-apoptotic protein

C) Untransfected BMK-DKO-^Canti-apoptotic cells, treated with BH3 mimetic diluted from stock 1000x in complete media.

Purpose: To measure if the BH3 mimetic changes the lifetime of the donor. Each compound must be tested separately. Minimally, include the highest concentration tested.

D) BMK-DKO-^Canti-apoptotic cells transiently transfected with plasmid (to express ^VBH3 protein of interest) and treated with DMSO diluted 1000x in complete media.

Purpose: To measure binding and generate a binding curve of ^Canti-apoptotic + ^VBH3. Include at least one well for each ^VBH3 protein tested.

E) BMK-DKO-^Canti-apoptotic cells transfected with plasmid (to express ^VBH3 protein of interest) and treated with BH3 mimetic diluted from stock 1000x in complete media. Include titration of BH3 mimetic in the plate.

Purpose: To measure the efficacy of BH3 mimetic to inhibit the interaction between the ^Canti-apoptotic & ^VBH3 proteins.

F) BMK-DKO-^Canti-apoptotic cells transfected with plasmid (to express the control non-binding mutant of acceptor-fusion (^VBH3-4E)) and treated with DMSO diluted 1000x in complete media.

Purpose: to use as a negative control for collisions.

7. Prepare live cell sample (3 days)

Ψ Note: *The sample plate of cells is prepared as we have recently described for traditional FLIM-FRET^{4,6,8} in a 384 well plate, only scaled up in the number of samples we can acquire per day (**Figure 1A**). All steps are performed in a biological safety cabinet, using aseptic techniques.*

*** Hint:** *Print plate-map (**Section 6**). Move column or row-wise across the plate and check off each transfection/treatment as they are added.*

? Troubleshoot: *If any changes occur while transfecting/treating cells (ie. two wells swapped) note the change in the platemap. Our analysis software enables correction of such mistakes by combining replicate data according to **what** is indicated as in the well on the individual platemaps, rather than using a single master plate-map.*

A) Seed cells (Day 1, ~1h)

- a. Trypsinize cells stably expressing mCerulean3-fused protein (Donor) of interest in a 10cm dish (70-90% confluent)
- b. Resuspend cells in 5mL DMEM complete media
- c. Count cells using a hemocytometer
- d. Dilute cells to 120 cells/ μ L, total volume ≥ 10 mL
- e. While continuously mixing cells by inverting tube, seed 25 μ L per well of a Corning CellCarrier 384 well plate. Final 2000 cells per well. Do not tilt the plate while seeding.

! Critical: *Remember to leave one column empty for standards.*

- f. Let stand at room temp for 15 minutes
- g. Return to incubator (37°C, 5% CO₂) for 12-24h

B) Transfect cells (Day 2, ~1h)

Ψ Note: See **Section 2.A.c** where we have already given a step-by-step description for preparing a transfection reaction for 2 wells. For each construct included in the screen determine the number of wells transfected and make a master transfection mix. We also include control reactions with transfection reagent + media without DNA added to “Untransfected” wells; Resulting in each well treated with transfection reagent. We always overestimate the number of wells (make enough for 2 extra wells) when making master mixes to guarantee enough to transfect all wells in platemap.

C) Treat cells (Day 2, ~2-3h)

3-5 hours after the transfection we routinely replace media with fresh DMEM complete (50μL per well) as shown in **Figure 1A**. For convenience, we selected this timepoint to treat cells with protein-protein interaction inhibitors.

- a. Thaw 1 aliquot of each compound prepared in **Section 5**
- b. In a 1.7mL tube, dilute each compound (or DMSO only) in complete media.

Ψ Note: Calculate volume needed based on plate design (**Section 6**). We

treat with 50µL per well in our 384-well plate format.

Ψ Note: *Maintain the same concentration of solvent DMSO per well for each concentration of compound tested. We diluted BH3 mimetics in DMSO, for 1:1000 dilution of each in complete media.*

- c. Remove transfection reaction media from each well with a pipette tip and replace media with 50µL of appropriate treatment.

Ψ Note: *We removed media from only one row at a time to minimize the time that cells are left without media.*

- d. Return to incubator (37°C, 5% CO₂) for 12-16h

8. Collect FLIM-FRET data on INO-FHS (1-2h setup + 24h automated data collection)

A) Add the following standards to empty wells in the sample plate (40µL per well):

- a. Venus protein standard curve
- b. mCerulean3 standard curve
- c. Quenched fluorescein
- d. 10nM fluorescein in 0.1M NaOH

B) Load imaging configuration (excitation wavelengths/filter settings), set environmental controls (5% CO₂ and 25°C), designate a location to save data, and align 384 well plate in NIS-Elements.

Ψ Note: *Step by step method for operation of our INO-FHS instrument, selection of imaging settings and acquisition time is described elsewhere⁶. We summarized steps for data acquisition in **Figure 1B**, with a few updates.*

C) Go to well with stable untransfected cells. Select 'fast scanning mode' (0.5s Frames Per Second (FPS)), focus on cells and align the pinhole manually by moving the x/y position of the pinhole by 1000 μm steps. Adjust focus if needed. Find the position where average counts are at maximum.

Ψ Note: *We find this method allows us to monitor changes in the confocal image while aligning the pinhole, which results in better quality images compared to when we align the pinhole using fluorescein as we previously described⁶.*

D) Go to well with 10nM fluorescein in 0.1M NaOH. Move focal plane into solution (100 μm above the focal plane of the cells). Select 0.055 FPS and acquire the image.

Ψ Note: *The purpose of this step is to ensure sufficient photon counts. When we optimized the imaging parameters⁶ we measured our 10nM fluorescein in 0.1M NaOH sample. From previous experience, an average of 10-13 counts per pixel is sufficient for data collection.*

? Troubleshoot: *If the observed count from standard fluorescein is much less than expected, go back to the "Untransfected" cells and retry the pinhole alignment. If this*

does not work, add fresh fluorescein to the plate; check that there is sufficient water on the objective and/or increase laser power to meet minimum expected counts.

- E) Once settings have been optimized and pinhole aligned save the configuration.
- F) Move back to the focal height of the cells.
- G) Collect, set, and save “DARK” counts as described⁶
- H) Move to quenched fluorescein sample. Collect, set, and save instrument response function (IRF) as described⁶
- I) Collect data for fluorophore standard curves (~10 minutes):
 - a. Move up 100µm (into solution)
 - b. In NIS Elements ‘WellSelection’: select the wells containing standard gradients of the donor (mCerulean3) and acceptor (Venus) purified protein.
 - c. In NIS Elements ‘GeneratedPoints’: select 2 fields of view per well
 - d. In INO-FHS acquisition software, check “autosave images” and “save to new file”.
Save the configuration file.
 - e. In NIS Elements click “INO-F-HS initialize” then “INO-F-HS Grab”. Then run automated data collection.
 - f. Once completed, move generated, ‘.TIFF’ files to a new subfolder called “StandardGradient_TIFFs”
- J) Move to a well with cells. Move down 100um then turn on autofocus. Set optimal focal height using fast scanning mode (0.5 FPS).
- K) In INO-FHS acquisition software, set frame rate to 0.055 FPS, check “autosave images” and check “save to new file” then save the configuration file.
- L) Collect data for the screen on INO-FHS (24h)

Ψ Note: *As we described recently⁶, we use NIS Elements software to acquire one field of view (FOV) per well of a 384 well plate sample then rescan the plate for a second FOV and so on. This minimizes the difference in acquisition time across the plate.*

- a. In NIS Elements 'WellSelection': select wells containing cell samples.
- b. In NIS Elements 'GeneratedPoints': select 1 field of view per well
- c. Repeat a. for WellSelection1, WellSelection2, etc., and input a single GeneratedPoint for each.

! Critical: *Be sure to select a different location for each FOV.*

Ψ Note: *Analysis required a minimum of four 200μm x 200μm images per well⁶. In general, we acquired 8-10 images per well for each screen.*

- d. Save NIS Elements Job file
- e. Run automated data collection (24h)

! Critical: *Generated, '.TIFFs' files are large. Make sure there is enough space to save the amount of data to be collected in your screen! Each file is 405 MB, thus for 8 fields of view, in a 384 well plate the total size is ~1.25TB (one replicate of a screen).*

9. Repeat steps 7-8 to acquire 2 more replicates

QUANTITATIVE FAST FLIM-FRET DATA ANALYSIS

SETUP FOR ANALYSIS

A) Install Software:

a. MATLAB R2020a

! Critical: *Data analysis requires MATLAB Add-on Toolboxes:*

- *Curve Fitting Toolbox*
- *Signal Processing Toolbox*
- *Image Processing Toolbox*

b. Microsoft Excel

c. INO Client Release_r10357 package:

- INO_F-HS_Acquisition
- INO_F-HS_Analysis
- INO_F-HS_BatchAnalysis

Ψ Note: *We used GraphPad Prism8 and CorelDraw X8 to plot heatmaps and figures displayed in this text. ROI selection done in INO-FHS software can alternatively be done using MATLAB or Python.*

Ψ Note: *Contact lead contact for access to INO_F-HS software package. To install the software you need to first run the vc_redist_x64.exe. This installs the C++ compiler used by the INO FHS. Then copy "DMGraph.dll" to your C:\Windows\SysWOW64 location. Finally,*

you need to register the "DMGraph.dll". This is done by running the "Register DMGraph.bat". Depending on your Windows 10 security settings, the .bat file may not be processed correctly. In that case, you will need to use the command line to register it manually. If there are any issues please the contact lead contact:

David.Andrews@SRI.utoronto.ca.

B) Download our "Quantitative FAST FLIM-FRET" analysis package from

https://github.com/DWALab/Osterlund_et_al_2021.git

The "Quantitative_Fast_FLIM_FRET_Analysis" package contains 2 folders:

"Replicate_Analysis" and "Combined_Reps Analysis".

C) Organize data as shown in **Figure 2A**. The main root folder here is called,

"Experiment"; each replicate subfolder is named, "Rep#_ YYYYMMDD". User gives the experiment name.

D) Create a "MasterPlatemap" in Microsoft Excel, following the template given

(See example used in our BCL-XL screen: <https://doi.org/10.5683/SP2/QN9C9R>).

A separate sheet is made for each replicate (Rep1, Rep2, etc.). The well ID is divided into column A (Row) and B (column B). For each well, the cell line (column C), transfection (column D), treatment (column E) and concentration of treatment (column F) are indicated clearly.

Ψ Note: *If there is a problem with running MATLAB analysis later on, usually it is due to a problem with the user's platemap.*

? Troubleshoot: *Check all these rules have been followed:*

- a. Use consistent naming conventions for each replicate (ie. if transfection is “V-BAD” in Rep1 user cannot use, “VBAD” in the second replicate).*
- b. Include DMSO-treated transfected and untransfected wells (used as controls). In the platemap column E, must fill treatment ID as “DMSO” and “untreated” respectively.*
- c. Be aware of special symbol use. As in the example, report concentration of drug treatment micromolar using the letter “u” instead of micro “μ”; MATLAB doesn’t recognize this symbol.*
- d. Make sure all wells included in the “platemap” are in the screen. Do not include empty wells or wells used for the fluorescent standards in this platemap.*
- e. Avoid using spaces when indicating cell lines, transfection, or treatment. A dash or underscore links words; for example instead of, ‘mC3 MCL-1’ use ‘mC3-MCL-1’.*

D) As shown in **Figure 2A** copy our, “**CombinedReps_Analysis**” folder to your main experiment folder and make copies of our “**Replicate_Analysis**” folder to each replicate subfolder.

ANALYSIS

Ψ Note: *The analysis is broken down into a series of steps (1-13) summarized in **Figure 2**. We run the “Replicate Analysis” package (steps 1-7) for each replicate (**Figure 2B**) then combine and analyze results in the “Combined Replicates Analysis” package (Steps 8-13) (**Figure 2C**). Final data may be plotted as*

heatmaps in a screen. In our associated manuscript (Science Advances), we used qF^3 analysis to compare a panel of BH3 mimetic drugs for disruption of several BCL-2 family protein-protein interactions in cells. As an alternative to following the step-by-step protocol below, follow our tutorial videos publicly available on YouTube:

<https://www.youtube.com/playlist?list=PLUiSJrzzg9voe5sjA57olbfOLAGlrHXRC>

The above link includes the Replicate Analysis (**Video 1**) then Combined Replicate Analysis (**Video 2**).

REPLICATE ANALYSIS

1. Compress INO TIFF files (time required depends on the quantity of data. Can take 1-2 days)

Ψ Note: The collected INO .TIFF files contain FLIM and hyperspectral cubes collected through raster confocal imaging. The INO-FHS software provides a data compression option that uses the LZW lossless compression to reduce stored image files. However, compression adds significant overhead to the collection time (an additional 2 hours for 4 FOV in a 384 well plate). Consequently, we created a simple graphical user interface GUI in MATLAB to compress data post-acquisition. The data compression reduces the size of collected images 15-30 times; an important consideration in collecting and storing large screen data. The compression of a large data set may take 1-2 days on a single desktop. We recommend starting this step while collecting the following experimental replicates.

A) In the qF^3 _Analysis > Replicate_Analysis, locate the folder:

1_CompressINO_TIFFs

B) Open and run 'Compress_INO_DATA_GUI_V2.m' in MATLAB. A pop-up window will appear containing three buttons.

a. Click on "Select Input Folder" and navigate to the path where the uncompressed TIFFs

are located.

- b. Click on “Select Output Folder” to select a directory where the compressed TIFFs should be stored.

Ψ Note: *The output folder must be different from the input folder to avoid overwriting original data. For consistency, we suggest compressing data and save into the INO_TIFFs_compressed folder (Figure 2A).*

- c. Click “Compress Data”

Ψ Note: *When compression is complete the status message will display:
“Data compression is complete”*

- C) Confirm that all files have been successfully compressed by comparing the number of files in the input folder to those in the output folder.

Ψ Note: *The script generates a log.txt file containing names of files that it could not compress. This rarely occurs, but if it does find the original .TIFF and copy directly to the compressed data location.*

- D) Delete folder of original .TIFF data.

2. Protein Gradient Analysis (takes less than 1 hour)

Ψ Note: *The second step in our replicate analysis is performed to extract relationship between photon counts and concentration. As described earlier, donor and acceptor protein gradients are imaged before every experiment.*

A) Move the compressed .TIFFs for the mCerulean3 and Venus protein gradient into a separate folder

B) Open the INO_F-HS_Analysis software and create the analysis configuration file:

a. Click “Load Data” and select any .TIFF file from the protein gradient

Ψ Note: *We will use this image to set up analysis parameters, which we can apply to the remaining protein gradient TIFFs (in Batch Analysis).*

b. In the General section, under “Lifetime Fit Algorithm”, select the “MLE-NP_fixed” algorithm.

Ψ Note: *This is an implementation of the Maximum Likelihood Estimation (MLE) algorithm provided by INO. In our screen we did not use this, rather we extract lifetime decays in .CSVs and use phasor analysis (later).*

c. In the “Fit Parameters” section, change Tau_1 to 4000. Fix the Tau_1 by clicking on the checkbox next to it.

d. In the “Fit Parameters” section, change A0 to 50 (initial estimate of the contribution of the unbound lifetime in fitting).

e. In the ROI section, check the box next to “Auto ROI”. In the drop-down menu

select, "NaiveQuadrantBinning".

Ψ Note: *The image will be split into 4 quarters displayed in the scan viewer. TCSPC decay and the Spectral profile for pixels belonging to each ROI will be combined to generate 4 global profiles. Each of these profiles will be used to measure average photon counts and spectral intensity per pixel.*

f. In the IRF section, click "Load" and select the 'IRF.IRF' instrument response function profile collected for that experiment.

g. In the calibration section click "Load" and select the "DARK.HSCalib" hyperspectral calibration profile collected for that experiment.

h. Click "Save Config" to save all selected parameters for analysis. Save as "QuadrantAnalysis". File type automatically recognized as .INI = configuration Settings.

i. Exit the INO_INO_F-HS_Analysis application

C) Open the INO_F-HS Batch analysis application and run batch analysis:

a. Click "Load Config" and load the created "QuadrantAnalysis".

? Troubleshoot: *The INO-FHS analysis software looks for a specific folder to save temporary data. If this folder does not exist in your computer directory, the software will leave that information empty causing an error. If there is an error loading the configuration file, open your saved analysis configuration file in a text editor such as Note Pad. Search for "LocalSaveCacheDirectory" and look for the values after the "=" sign. If this value is empty add two single quotations '' to indicate that it is*

an empty string or path.

- b. Click “Load Folder” and select the path where the protein gradient .TIFFs are stored.
- c. Click “Save Config” and designate a path to save a copy of the configuration file.

Ψ Note: *Can save over the original QuadrantAnalysis configuration file.*

- d. Click “Save Folder” and designate a path to save the generated analysis results.

Ψ Note: *Here, results will be saved to a new folder “Results_YYMMDDTime” saving the date and time (military hour, minutes, seconds) that the analysis was run.*

For consistency we suggest saving results within Replicate_Analysis> folder:

2_ProteinGradientAnalysis.

- e. Click “Process Batch” to start the batch analysis process.

Ψ Note: *A pop-up window should appear showing progress. For each .TIFF analyzed, two files will be generated in the Results_YYMMDDTime folder: a TIFF file containing the segmentation maps of the generated ROIs and a .CSV file containing extracted data for each of interest (ROI) including ROI size, number of photons, MLE fitting profile estimates, number of iterations the MLE required to converge to a solution, TCSPC time-bin resolution, IRF profile, spectral wavelengths detected and spectral profile. Next, we run a MATLAB script to extract the standard*

curve data from these .CSV files.

D) In the “Protein Gradient Analysis\ 2_MATLAB_ExtractPixel-wise counts” folder open our “LoopThroughPurifiedProteinCSV.m” script in MATLAB.

- a. Copy the directory path to the “Results_YYMMDDTime” folder generated from the batch analysis of protein gradient image data. In MATLAB, paste this directory in the path parameter.
- b. Run “LoopThroughPurifiedProteinCSV.m”. In the inputted path directory an excel file named, “combined_gradient_results.xls” will be generated as results are exported.

Ψ Note: *Combined_gradient_results.xls contains three columns: the well ID, the average photon count per ROI at Time 0 (T0) in the lifetime decay (FLIM channel) and the average intensity measured at 530nm in the spectral channel.*

E) In Microsoft excel, determine the relationship between intensity and concentration using mCerulean3 and Venus protein gradients.

- a. Input the mCerulean3 and Venus concentrations corresponding to each Well_ID.
- b. Plot concentration of mCerulean3 (x-axis) versus photon counts (column B, y-axis) for the donor (mCerulean3) protein gradient.
- c. Plot concentration of Venus (x-axis) versus hyperspectral counts (Column C, y-axis) the acceptor (Venus) protein gradient.
- d. Fit data plotted in b and c to a straight line. The slopes of the line of best fit will be used later in the analysis.

Ψ Note: We fix the intercept to 0,0 and fit data that falls in a range of intensities where the relationship is linear. On the INO-FHS system, we determined this range in pilot experiments: imaging purified protein gradients with configuration settings that we had selected for screening⁶. We collected data for mCerulean3 and Venus protein concentrations up to 50μM. We observed saturation in the TCSPC channel for high concentrations of mCerulean3 and no saturation of the hyperspectral channel (DNS). If the image has saturated pixels then the relationship between counts/concentration cannot be linear. Thus, we selected a range of protein concentrations well below the point of saturation that we could expect to observe a linear relationship for both proteins (0-8μM mCerulean3 and 0-50μM Venus) in future experiments. We do not extrapolate this relationship beyond the concentrations of our purified fluorophores.

3. Select Subcellular ROIs (time required depends on the quantity of data. Can take 1-2 days)

Ψ Note: Collected INO .TIFF data are analyzed similarly to the protein gradient analysis using the INO-FHS analysis and INO-FHS batch processing software. Here, we identify subcellular regions of interest (ROIs), bin pixels within each ROI, and extract data. We selected the segmentation algorithm compiled as a dynamically linked library (DLL) written and compiled using Visual Studio C++ 2015. The DLL is loaded as a plugin (Client Release_r10357>INO_FHS_Confocal_Microscope>bin>ProcessPlugin). The INO software permits the user to define parameters used in the analysis, stored as a text file. Parameters such as the size of the Laplacian of a Gaussian filtering kernel, segmentation using FLIM or hyperspectral channel, as well as the minimum ROI size allowed. The saved FLIM-Hyperspectral cubes are saved into a multipage tiff format. The DLL has access to loaded FLIM and Hyperspectral cube pointers in memory. The segmentation DLL utilizes Open Computer Vision (OpenCV 3.4.1) library to perform image filtering and multi-seed watershed segmentation. Example segmentation is shown in **Figure 3**.

- A) Open the INO_F-HS_Analysis software and create an analysis configuration file
- Click “Load Data” and select any .TIFF file from the screen
 - In the General section, under “Lifetime Fit Algorithm”, select the “MLE-NP_fixed” algorithm.
 - In the “Fit Parameters” section, change Tau_1 to 3800 (initial estimate of the untransfected lifetime in picoseconds) and check the box next to Tau1. Change A0 to 50 (initial estimate of the contribution of the untransfected lifetime).

Ψ Note: *Initial estimate is just a starting point for the fitting. Checking the box fixes a parameter.*

- In the ROI section, check the box next to “Auto ROI”. In the drop-down menu select, “NehadWatershedSegmentation”.

Ψ Note: *This will segment the cells into ROIs. Clicking on any ROI will display the TCSPC and the spectral profile. Note that some ROIs selected may appear to segment background pixels, however, these ROIs will not meet the minimum number of photons required for fitting and will not be exported in analysis.*

- In the IRF section, click “Load” and select the ‘IRF.IRF’ instrument response function profile collected for that experiment.
- In the calibration section, click “Load” and select the “DARK.HSCalib” hyperspectral calibration profile collected for that experiment.
- Click “Save Config” to save all selected parameters for analysis. Save as

“WatershedSegmentation”. File type .INI = configuration settings

h. Exit the INO_INO_F-HS_Analysis application

B) Open the INO_F-HS Batch analysis application and run batch analysis for the entire screen:

- a. Click “Load Config” and load the created “WatershedSegmentation”.
- b. Click “Load Folder” and select the location of the INO TIFFs_compressed folder.
- c. Click “Save Config” and designate a path to save a copy of the configuration file.
- d. Click “Save Folder” and designate a path to save the generated analysis results.

Ψ Note: *For consistency, we suggest saving results to Replicate_Analysis> folder:*

3_SelectROIs

e. Click “Process Batch” to start the batch analysis process.

4. Combine CSV data belonging to the same well (time required is less than 20 minutes)

Ψ Note: *INO_analysis software generates a comma-separated value (CSV) and a .TIFF file (saved ROI segmentation) for each image analyzed. Here, since multiple fields of views are acquired per well, we use command prompt to combine CSV files belonging to the same well into a “combined_csv”. Command prompt/bash scripts are currently most efficient for combining large CSV files. The generated CSV files contain numerical values represented using an ASCII format and they tend to be larger than the typical binary format. Combined CSV files can reach up to 160 Megabytes. For a single screen repetition, the size of the generated combined CSV files can reach up to 50 Gigabytes.*

A) In the Results folder of batch analyzed screen data, move all the .CSV files to a separate subfolder, “CSVs”.

B) Open the “Command Prompt” application

Ψ Note: *In Windows, click on Start Menu and type “cmd” to find it.*

C) Change to the CSV file directory by typing “cd” followed by a space and directory path (ie. cd C:\Data), then press ‘ENTER’ to execute the command.

Ψ Note: *Right-click to paste in command prompt.*

D) type “dir” followed by hitting the ‘ENTER’ key

Ψ Note: *command prompt will generate a list of files in the selected directory at this step.*

? Troubleshoot: *If you do not see a list of filenames, redo **4.C)-D)**. If again you do not see a list of filenames, we suggest making a temporary copy of your “CSV” folder to the desktop. Then redo **4.C)-D)**, inputting the desktop>CSV folder directory instead. Delete temporary copies after **F)**.*

E) Copy and paste the following line into the command prompt window:

```
For %i in (A,B,C,D,E,F,G,H,I,J,K,L,M,N,O,P) DO For /L %j in (1,1,24) Do copy /b WellID_%i%j-*  
combined_%i%j.csv
```

Ψ Note: *This command generates well IDs in order. It identifies the files that have the same IDs and copies their content to a single file. You will see “Combined_WellID” files generated in the location of your CSV files ie. Combined_A2.*

F) Move all “Combined_CSV” data files to a separate subfolder.

Ψ Note: *For consistency, we suggest using the Combined_CSVs folder located:*

Replicate_Analysis>4_CombineDataPerWell.

5. Filter CSV data and extract binding curves (time required = usually 2-4h)

Ψ Note: *In this step, slopes of our standard curves (Analysis step 2) are used to convert intensities to concentrations and determine the fractional change in angular frequency (Δ): a value that is linearly related to bound fraction, by phasor analysis of lifetime per ROI (see associated manuscript for more details). **Figure 4A** is an overview of the tasks completed in this step. The MATLAB script requires the MasterPlatemap (created earlier).*

! Critical: *This script uses the G-Factor determined as described in Preparatory Experiment Step 2: Determine G factor. The G-Factor must be updated if the experiment is being carried out on a different microscope or if the filter settings have been changed on the INO-FHS. To update the G-Factor parameter open the “CurveExtractionAndShift.m” script located in Replicate_Analysis> 5_FilterData_ExtractRawCurves and change the parameter, “G_FACTOR” to the value determined for the instrument/settings used for the screen. As noted earlier, instruments that have a time-gated acceptor channel will not require the G-factor correction at all. Contact author, Nehad Hirmiz for advice on how to adapt this script for such instruments.*

A) Copy the full path to the MasterPlatemap, for example, C:\Data\MaterPlateMap.xlsx.

B) In MATLAB, open and run the FilteredDataExportationSonstantTx.m script located in

Replicate_Analysis> 5_FilterData_ExtractRawCurves as follows:

- a. Update the ‘excel_file’ parameter by pasting the full path to the MasterPlatemap and update the ‘excel_sheet’ parameter to indicate which sheet to reference. For

example, in our MasterPlatemap sheets are labelled “Rep1”, “Rep2”, etc.

Ψ Note: *Need to match the sheet name in the MasterPlatemap exactly.*

b. Indicate which cell line(s) exist in the plate by updating the cells parameter.

For example, cells = {'mC3-MCL-1'},{'mC3-BCL-2'},{'mC3-BCL-XL'}}

Ψ Note: *The name {here}, must exactly match how the name appears in your MasterPlatemap, column C.*

c. Copy the path to your Combined_CSV folder (Analysis step 4G). Then in MATLAB update the parameter: path_to_combined_csv

Ψ Note: *paste path here 'path\', leaving the \ at the end.*

d. From Analysis Step 2, get slopes of Venus and mCerulean3 gradients and update the parameters: venus_gradient_slope and mc3_gradient_slope

e. Run the script.

! CRITICAL: *Let the script run to completion before opening any exported file in Microsoft Excel.*

Ψ Note: *By default, the results are saved into the same directory as the script in a*

subfolder, "RawCurves". Results for each well in the platemap are saved as:
 combined_WELL_ID_filtered_raw.csv (example combined_A2_filtered_raw.csv).
 Each file contains calculated Venus concentration, mCerulean3 concentration, free
 Venus concentration, bound mCerulean3 fraction, and FRET activity per ROI.
 Columns A-E data were determined by phasor analysis of lifetime. To generate an unbinned
 binding curve with raw data: plot column C versus column D (Δ) or versus column E
 (FRET efficiency). We found the phasor lifetime analysis more reliable than the
 double exponential fit performed in the INO-Batch Analysis software (see Analysis
Step 3A.b-c) since the phasor analysis does not require user input. However,
 the INO fit analysis data is still exported in columns F-J, in case useful. The Free
 Venus concentration (column F) recalculated using the fraction of mCerulean3 with
 a lifetime shorter than τ_1 (where τ_2 is unfixed) as determined from the double
 exponential fit (column G), corresponding FRET efficiency (column I) and
 χ^2 to estimate the goodness of fit (column J). Finally, we export the
 acceptor:donor intensity ratio (column H), which may be used to generate
 traditional, non-quantitative FLIM-FRET binding curves if desired. All this is
 exported for each ROI to provide users with many options to examine/filter data.

Ψ Note: In addition, this script exports the phasor coordinates for each
 untransfected, treated well in the platemap (**Figure 4A**). This data is exported as,
 "Untransfected_Phasor_Coordinates.xlsx" in the RawCurves subfolder.

6. Get Lifetime for Untransfected controls (time required= 5 mins)

Ψ Note: In Analysis Step 5, we extracted the phasor coordinates of the unbound lifetime (τ_1 in phasor plot) for each Untransfected treated control to use in determining Δ for corresponding treated, transfected wells. Some treatments may affect the donor lifetime, which can lead to errors in the 2-component analysis. For example, see the change in τ_1 with ABT-199 treatment of BMK-DKO cells expressing mCerulean3-BCL-2 (**Figure 4B**). In this step, we convert the exported phasor coordinates from Step 5 to a lifetime (ns) to examine the effect of each drug on the donor lifetime.

A) In folder Replicate_Analysis> 6_Get_LifetimeUntransfectedControls> open MATLAB

script: "GetLifetimeFromPhasorCoordinates.m"

a. Copy full path to the "Untransfected_Phasor_Coordinates.xlsx" file generated in the previous step then in MATLAB, update path_to_phasor_coordinate parameter

b. Run the script

Ψ Note: Results are exported in the same location as the script with filename:

UntransfectedLifetimeFromPhasor.xlsx. This file includes only untransfected wells from the platemap. The Well ID, Cell line, Treatment, and Concentration, and average lifetime (nanoseconds) per well are given in columns (A-E, respectively).

Ψ Note: Later in step 13, we combine 3 or more replicates to examine the effect of treatment on donor lifetime. If a compound directly changes donor lifetime $> 0.2\text{ns}$ then remove from published screen data. For example, we observed ABT-199 treatment has a direct effect on mCerulean3 lifetime in BMK-DKO cells expressing mCerulean3-BCL-2 in our associated manuscript. As a result, we observed unexpected trends in the data suggesting errors in the 2 component analysis. Hence, we considered the qF^3 binding curves for ABT-199 preliminary and removed them from the main paper.

7. Bin binding curve data (time required = 30 mins)

Ψ Note: *In this step, we filter, and bin the raw binding curve data generated in Analysis step 5 (Figure 4C). This will allow us to combine binding curves from multiple replicates. This script also generates a sheet, "Donor_Concentration_Table.xlsx" containing mean donor concentration in each well, which will be used in later steps to examine the variation of donor expression across the plate.*

A) In the Replicate_Analysis> 7_Bin_CurveData_FreeAcceptor folder, open and run the MATLAB script: "UnshiftedRawCurveBinning.m script.m" as follows:

a. Update the 'excel_file' parameter by pasting the full path to the MasterPlatemap and update the 'excel_sheet' parameter to indicate which sheet to reference. For example in our example MasterPlatemap sheets = "Rep1", "Rep2", etc.

Ψ Note: *Need to match the sheet name in the MasterPlatemap exactly.*

b. Copy the directory to the "RawCurves" folder containing .CSV files exported in Analysis Step 5. Input the path in MATLAB.

c. Modify filters if desired. Open MATLAB script, "BinRawUnshiftedData.m" and update parameters: B (Δ) and FA (free acceptor).

$B = B(FA < 50 \ \& \ mC3 > 1 \ \& \ mC3 < 3);$

$FA = FA(FA < 50 \ \& \ mC3 > 1 \ \& \ mC3 < 3);$

These lines specify that we keep ROIs that have free acceptor less than 50 μM and that have mCerulean3 concentration between 1-3 μM .

Ψ Note: *Default script filters data to keep ROI data that have a mCerulean3 concentration from 1-3μM and a Venus_{Free} concentration less than 50μM.*

d. Modify Bins if desired. Open MATLAB script, “BinRawUnshiftedData.m” and update parameter: bins1

Ψ Note: *Default bins for quantitative curves are indicated in bins1 parameter:*

[-1,1,2,3,4,6,8,10,12,16,20,30,40,50]

Default bins for Acceptor:donor intensity curves are indicated by the bins2 parameter.

e. Run the script. Results will be exported to three subfolders in the same location as the script:

i) AD_FRET_Binned_Curves subfolder

Ψ Note: *In this folder, we extract traditional FLIM-FRET binding curves for each well. The data is binned by Acceptor:Donor intensity ratio and for each bin, median FRET efficiency, standard deviation, and number of points are exported. While the script bins and exports these data we did not use these results in our associated publication.*

ii) INO_Binned_Curves subfolder

Ψ Note: *In this folder, we extract the binned binding curves generated from the*

INO-FHS fitting analysis. The data is binned by $Venus_{Free}$ concentration and for each bin, median Δ , standard deviation and the number of points per bin are exported. While the script bins and exports these data we did not use these results in our recent publication.

iii) Phasor_Binned_Curves subfolder

Ψ Note: *In this folder, we extract the binned binding curves generated from the Phasor lifetime analysis (step 5). The data is binned by $Venus_{Free}$ concentration and for each bin, median Δ , standard deviation and number of points are exported and used in our associated publication.*

ANALYSIS OF COMBINED REPLICATES

Ψ Note: *For three or more biological replicates of the screen, complete the “Replicate Analysis” (Figure 2B, steps 1-7). The next part of the analysis “Combined Replicates Analysis” package (Steps 8-13) are illustrated in Figure 2C. As an alternative to following the step-by-step protocol below, follow our tutorial videos in Combined Replicate Analysis (Video 2).*

8. Combine binned data for multiple replicates

A) In CombinedReps_Analysis> 8_CombineBinnedReps folder, open MATLAB script,

“CombineBinnedFilterDataFor3Reps.m” to combine 3 replicates

OR

"CombineBinnedFilterDataFor4Reps.m" to combine 4 replicates.

! CRITICAL: *The bins parameter in this script must be the same as the bins used in the Replicate Analysis. If bins were changed earlier in analysis **Step 7. A) d** then update the bins parameter here.*

a. Update the path to the MasterPlatemap (excel_file parameter)

Ψ Note: *Plate maps for repeat experiments do not need to be identical. The script uses the first repetition "Rep1" sheet as a reference and searches for the identical Cells/transfection/treatment/concentration in the remaining repetitions.*

b. Copy each path to binned data generated in Replicate Analysis Step 7 and input parameters: path_to_binned_data1, path_to_binned_data2, path_to_binned_data3

c. Input the path to two folders where data will be exported:

i) The combined binned data (combined_data_export_path).

Ψ Note: *Rep1, Rep2, Rep3, Rep4 binned data are pasted into one excel file.*

ii) The average of the binned data (average_binned_export_path).

Ψ Note: *For consistency we suggest to save data to CombinedReps_Analysis> 8_CombineBinnedReps subfolder, "CombinedBinned_Reps", "AveragedBinned_Reps".*

- d. Run the script. For each transfected well in the platemap, a file will be generated containing the combined binned data from 3+ replicates.

9. Fit combined, binned binding curves (Time required= ~1h)

Ψ Note: Refer to our associated publication for rationale and details behind screen analysis and fitting criteria. In summary, see **Figure 2C**. In order to identify a true protein-protein interaction, each binding curve must be compared to a collisional (negative) control. The script constructs a table of control transfections (DMSO treated only) and finds the negative control for each. We then set the minimum difference between the test and negative control for continued screen analysis: the difference in median Δ between the untreated positive and negative controls ($\Delta\beta$) must be larger than 0.05. If $\Delta\beta > 0.05$ then we calculate % Resistance to drug treatment, as described. However, binding does not mean there is sufficient data to fit for the determination of K_d . So, we established a second criterion for fitting K_d : the shape ratio ($sRatio$) must be greater than 2. This is a non-parameterized approach to describe the shape of the binding curve, without fitting. If $sRatio$ is >2 then we consider that the binding curve has sufficiently plateaued to fit data with a single-site binding Hill slope equation (fixed Hill Slope=1) and determine K_d .

A) In CombinedReps_Analysis>9_Fit_CombinedCurves open MATLAB script,

“AutomatedKdExtractionRepsAllPoints.m” and run as follows:

- a. Update the path to the MasterPlatemap (excel_file parameter)

Ψ Note: The script uses “Rep1” sheet as a reference to identify the positive and negative controls for each experiment.

- b. Input path to the folder containing combined, binned CSVs (parameter= path_to_binned_csvs) generated analysis step 8: *CombinedReps_Analysis*>

8_CombineBinnedReps> CombinedBinned_Reps.

c. Input cell line(s) exactly as they appear in the MasterPlatemap. Paste each name

{here} ie. cells = {{'mC3-BCL-2'}, {'mC3-BCL-XL'}}.

d. Input positive/test transfection(s) exactly as they appear in the MasterPlatemap.

Paste each name {here} ie. = {'V-BAD'}, {'V-tBID'}, {'V-BIML'}.

e. Input corresponding negative/collisional controls exactly as they appear in the

MasterPlatemap. Paste each name {here} ie. = {'V-BAD-4E'}, {'V-tBID-4E'},

{'V-BIML-4E'}.

! Critical: *A negative control must be indicated for each positive control and must be listed in matching order.*

Ψ Note: *The same negative control can be used for multiple test wells.*

ie. if positive controls are = {'V-BIML'}, {'V-BIML-mutant1'}, {'V-BIML-mutant2'}

the corresponding negative control "V-BIML-4E" should be inputted three times.

Negative controls = {'V-BIML-4E'}, {'V-BIML-4E'}, {'V-BIML-4E'}.

f. Run script. At the beginning of the analysis, each positive control binding curve will displayed in a pop-up window as shown in **Figure 5A** appears for the user to review.

Ψ Note: *The script estimates the point of binding saturation based on the median of the last 6 points in the binding curve (median Δ of the points with the highest $Venus_{Free}$ concentration). The estimated Saturation point is plotted as a blue line.*

- i) If the user approves the estimated saturation parameter, then hit “ENTER” to continue with the next dataset to review.
- ii) If the saturation parameter is not correctly estimated, the user can override and input the saturation parameter by typing, “-1” in the command window then manually entering the saturation parameter (ie. 0.2), then hitting ENTER to continue analysis.

Ψ Note: *This script exports the saturation parameters for all of the controls in the file “SatParameters_CombinedBinnedReps.csv”. This saves the fitting parameters, including any user input, for future replication of fitting analysis and for plotting the fit later in step 10. Note that the fitting parameters determined using these positive control wells are subsequently fixed in fitting all wells with the matching transfection as well as for fitting the corresponding negative control. The script exports full plate analysis (“CombinedBinnedReps_Results.xlsx”) to the same location as the combined, binned data: CombinedReps_Analysis> 8_CombineBinnedReps> CombinedBinned_Reps folder. This file contains columns A-F copied from the MasterPlatemap for data navigation. In columns G-P we export:*

G: Sum the number of ROIs analyzed in total to make up the curve)

H: Sum the number of binned points in the combined, binned curve)

I: Maximum $Venus_{Free}$ (μM) concentration of the binned points in the combined, binned curve

J: Cumulative Sum of the area under the curve

K: Median Δ of binned points that fall within 10-20 μM on the combined, binned curve

L: % Resistance to treatment (drug), calculated as previously published⁴

M: Shape Ratio, calculated as described earlier

N: Kd ($\mu\text{M Venus}_{\text{Free}}$), calculated from fitting combined, binned data to a one site specific binding Hill slope equation

O: Lower confidence interval of fitting Kd ($\mu\text{M Venus}_{\text{Free}}$)

N: Upper confidence interval of fitting Kd ($\mu\text{M Venus}_{\text{Free}}$)

10. Plot combined binned data (Time required: ~10-20 mins)

Note: In interpreting heatmap results, sometimes it helps to open the binding curve to examine the data and fit. To save time, in this step we run a script that plots each binding curve in the platemap (combined, binned data exported in step 8). Usually, we want to compare the result to the positive and negative control, so the script is designed to find the corresponding control and plot them on the same graph, as shown in **Figure 5B**. Exported plots display the upper and lower confidence interval for the fit of each curve using a shaded area. Blue shade for positive control, gray shade for the negative control, and red shade for the binding curve of interest (WellID + cells + transfection + treatment saved in the title of the graph). In the “simple curve plotting” algorithm we apply the same thresholds ($\Delta\beta < 0.05$ or $s\text{Ratio} < 2$), and use the same saturation parameters (including user-input), so the displayed fit matches what was done in step 8 to extract Kd.

A) Go to the CombinedReps_Analysis> 8_CombineBinnedReps>CombinedBinned_Reps folder and open the “SatParameters_CombinedBinnedReps.csv”, which was exported in Analysis Step 9. Save as an excel file, “SatParameters_CombinedBinnedReps.xlsx”.

B) In the CombinedReps_Analysis>10_Plot_CombinedCurves folder, open

“SimpleCurvePlotting.m” in MATLAB

a. Input the full path (“ie. C:\Data\CombinedReps_Analysis\8_CombineBinnedReps\CombinedBinned_Reps\ SatParameters_CombinedBinnedReps.xlsx”) to the

saturation parameter table to update the satpath parameter.

b. Copy the directory to the “CombinedBinned_Reps” folder (**Step 8.A.c.i**)

and update the path_to_binned_csv parameter (For example,

C:\Data\Analysis\8_CombinedBinnedReps\CombinedBinnedReps)

Ψ Note: *file must be named SatParameters_CombinedBinnedReps.xlsx, otherwise need to update the “satfile” parameter as well*

c. Update the path to the MasterPlatemap (excel_file parameter) and indicate the name of the sheet used as a reference. By default, this is, “Rep1”

d. Update experimental conditions similar to how we did in Step 9.

i) Input cell line(s) exactly as they appear in the MasterPlatemap. Paste each name {here} ie. cells = {{‘mC3-BCL-2’},{‘mC3-BCL-XL’}}.

ii) Input transfection_reference parameter (positive/test transfections) exactly as they appear in the MasterPlatemap.

Paste each name {here} ie. = {{‘V-BAD’},{‘V-tBID’}}.

iii) Input negative_reference parameter (negative/collisional controls) exactly as they appear in the MasterPlatemap.

Paste name {here} ie. = {{‘V-BAD-4E’},{‘V-tBID-4E’}}

! Critical: *A negative control must be indicated for each positive control and must be listed in matching order.*

e. Input a directory to save plotted graphs, update the path_to_final_graphs parameter

Ψ Note: For consistency, we save graphs in the location:

CombinedReps_Analysis>10_Plot_CombinedCurves subfolder, "Combined_Curves"

f. Optional * select format for export (at end of the script)

Ψ Note: Default script exports a .jpg and .eps file for each graph. If another format is desired, then modify the line towards the end of this script, 'saveas'

g. Run script.

11. Determine average donor concentration per well (Time required: 5 mins)

Ψ Note: Detected binding affinity depends on the amount of available donor and acceptor labelled proteins in each ROI. We restrict our analysis to ROIs that have a limited range of donor labelled protein expression to ensure consistency across the plate map and between replicates. We perform this step to check that expression of the donor is consistent across the screen plate and that no treatment has a dramatic effect on donor concentration. Recall that in Replicate_Analysis step 7, we exported a table containing the average donor concentration per well for each replicate in file "Donor_Concentration_Table.xlsx". Here we combine these data for all replicates: the script identifies what is in the well from Rep1 then it finds the corresponding treatment in Rep2, 3 and 4 to combine the data.

A) In folder CombineReps_Analysis > 11_GetAvgDonorConcentrationPerWell, open

"GetAverageDonorConcentration_3Reps.m" in MATLAB

Ψ Note: If you have 4 replicates, "GetAverageDonorConcentration_4Reps.m"

a. Input path to the folder containing for each *Donor_Concentration_Table.xlsx* from

replicates 1-4, updating parameters: path_donor_table_1, path_to_donor_table2, path_to_donor_table3 and path_to_donor_table4

b. Run script.

Ψ Note: *The results will be exported in the same folder. Two files will be generated: the first, "Average_Donor_Concentration.xlsx" using the list format contains data in list format, where columns A-F were copied from the MasterPlatemap for data navigation and in columns G-J we export:*

G: Mean of replicates of the average mCerulean3 concentration (μM) per well

H: Standard deviation of column G

I: Median of replicates of the average mCerulean3 concentration (μM) per well

J: Mode of replicates of the average mCerulean3 concentration (μM) per well

The second file exported, "Average_Donor_Concentration_Maps.xlsx" takes the same data and transforms it to a platemap array for plotting heatmaps.

12. Generate Heatmaps for the entire screen (Time required: 5 mins)

Ψ Note: *In this step, we convert results (exported in Analysis Step 8) from a list format to a 384 well platemap array. This is ideal for plotting heatmaps, viewing and interpreting results from large screens.*

A) Navigate to CombinedReps_Analysis > 12_GenerateHeatmap and open script,

"GenerateParameterMap.m" in MATLAB

a. Copy path to the "CombinedBinnedRepsResults.xlsx" file saved in step 8 then update excel_path parameter

Ψ Note: *If the filename/sheet name has been modified the user must update the script.*

b. Run Script. Wait for the message indicating analysis completed.

Ψ Note: *the script converts the data from a list to a platemap array format, saved as "GeneratedHeatMapsFinal.xlsx" in the same location as the excel_path.*

B) (Optional) navigate to CombinedReps_Analysis > 12_GenerateHeatmaps and open "PLOTHeatmap.m" script designed to plot Heatmaps in MATLAB

Ψ Note: *Heatmaps displayed in our associated paper were plotted in Graphpad Prism. This is an alternative script to generate heatmaps.*

a. Open resulting data, "GeneratedHeatMapsFinal.xlsx" exported in platemap array format. For whatever value you wish to plot a heatmap of (ie. Kd, number of points per well, Venus expression per well, %Resistance etc.) copy data from excel.

Ψ Note: *User can copy the entire 384 well plate, a section of it, or rearrange data for plotting.*

b. After kd_map, paste data [[here](#)] (within the brackets in place of example data)

c. Select color map (myColorMap parameter) and scale (parameter caxis=

([min_value,max_value]))

Ψ Note: The default colormap is “Jet” divided into 100 segments.

d. Run the script. A pop up window should appear with the resulting heatmap.

? Troubleshoot: You may see the following, “Error using vertcat Dimensions of arrays being concatenated are not consistent”. This error occurs due to the presence of empty wells in the pasted data. To fix this, make sure each cell copied contains a numerical value. To get around this, we manually fill in any empty well with some known outstanding numerical value. Make sure to keep track of changes in data to avoid confusion. For example, if data was insufficient for fitting, then the value for Kd will be exported as ‘NaN’ in Analysis step 9. In converting to a platemap array, all wells marked “NaN” will be empty. We suggest filling empty wells with a nonsensical value; for example, a Kd of ‘-1000’ is easy to recognize and remove (make appear blank) before publishing final heatmaps.

13. Investigate the Lifetime for Untransfected Cells

Ψ Note: In Replicate Analysis **Step 6b** as we exported lifetime data for all untransfected wells in the file: *UntransfectedLifetimeFromPhasor.xlsx*. In this step of the analysis, we combine the results from multiple replicates based on finding what is in each row in MasterPlatemap Rep1 (Untransfected cell line, treatment, and concentration) then searching for the corresponding data in Replicates 2,3,4. This method of combining data is useful to account for any change in Well locations between replicates. If there were no changes in the platemap design between replicates, the user can easily combine data by hand.

A) Navigate to CombinedReps_Analysis > 13_Combined_UntransfectedLifetimeReps and open “CombineLifetimeInfofrom_3Reps.m” in MATLAB

Ψ Note: *for 4 replicates use “CombineLifetimeInfofrom_4Reps.m”*

- a. Update the full paths (path_excel1, path_excel2, path_excel3, and path_excel4) to the “UntransfectedLifetimeFromPhasor.xlsx” file generated for each replicate respectively.
- b. Run the script. The results will be saved in the same location as the script.

Ψ Note: *By this method, we plot data shown in Supplementary Figure 5 of the main associated text. We identified that ABT-199 has a significant impact on the mCerulean3 donor lifetime and all binding curves probed by this compound were subsequently removed after failing to pass quality control standards.*

Troubleshooting

Noted in procedure

Time Taken

Noted throughout procedure and in Figures for main steps of our quantitative Fast FLIM-FRET (qF3) method.

Anticipated Results

We displayed K_d values as a read-out for our BH3 mimetics screen. However, many other useful results are exported from the fitting analysis of the final Combined_Binned curves. As an example here we

provide additional data generated for a subset of the protein-protein interactions screened in Figure 5 of our associated publication (Science Advances).

From Step 12_GenerateHeatmaps filename: *GeneratedHeatMapsFinal.xlsx*

and

From Step 11_GetAvgDonorConcentrationPerWell: *Average_Donor_Concentration_Maps.xlsx*

Taking these files as an example, from our screen in BMK-DKO cells expressing CBCL-W as the donor ($n > 3$ biological replicates), example data is shown in **Figure 6**. The number of ROIs (**Figure 6A**) for each combined binned curve can tell the user if the treatment might affect the number of cells (unknown toxicity) or the number of transfected cells (treatment affecting the stability of acceptor?). The Average concentration of mCerulean3 per well should be unaffected by treatment/transfection since the donor is stably expressed (**Figure 6B**). If the number of ROIs or concentration of mCerulean3 per well is drastically reduced this can alert the user to take a closer look at those corresponding images to determine why. The maximum concentration of VenusFree (μM) (**Figure 6C**) tells the user the range of the data in each binding curve. In our analysis, we require a minimum of $15\mu\text{M}$ VenusFree for fitting, therefore the dataset: VNOXA + $20\mu\text{M}$ S-64315 maximum = $13\mu\text{M}$ is insufficient, and appears as "X" in subsequent heatmaps. The cumulative sum of the area under the curve (**Figure 6D**) can be used to visualize binding versus no binding, however, is highly dependent on the maximum Δ . Heatmaps shown in colour, were most useful for visualizing drug effect on an interaction, where blue indicates binding and red indicates no binding or effect of an inhibitor. Shape Ratio (area under the isotonic fit of the data divided by the area over the isotonic fit of the data, within the bounding box of that dataset) (**Figure 6E**) is independent of the maximum Δ and tells the user whether the data is more linear (sRatio = 1 for perfect line) or curved (sRatio > 2). The median $\Delta\omega$ of points between $10\text{--}20\mu\text{M}$ VenusFree (**Figure 6F**) is useful to understand the FRET efficiency between the two proteins of interest (low $\Delta\omega$ = low FRET). Combining **Figure 6 C,E,F** the user can visualize the binding curve. Data in Fig 6f is used to calculate 1) $\Delta\beta$ (difference in $\Delta\omega$ between untreated positive and negative controls) and 2) % Resistance to drug treatment as previously described⁴; the less Resistant the more effect of the drug. If $\Delta\beta < 0.05$ then % Resistance is not calculated, and thus appears as "-" in the example heatmap for VNOXA (**Figure 6F**). Finally, if $\Delta\beta > 0.05$ and if sRatio > 2 (for untreated positive control), and Dissociation constants are determined (**Figure 6G**).

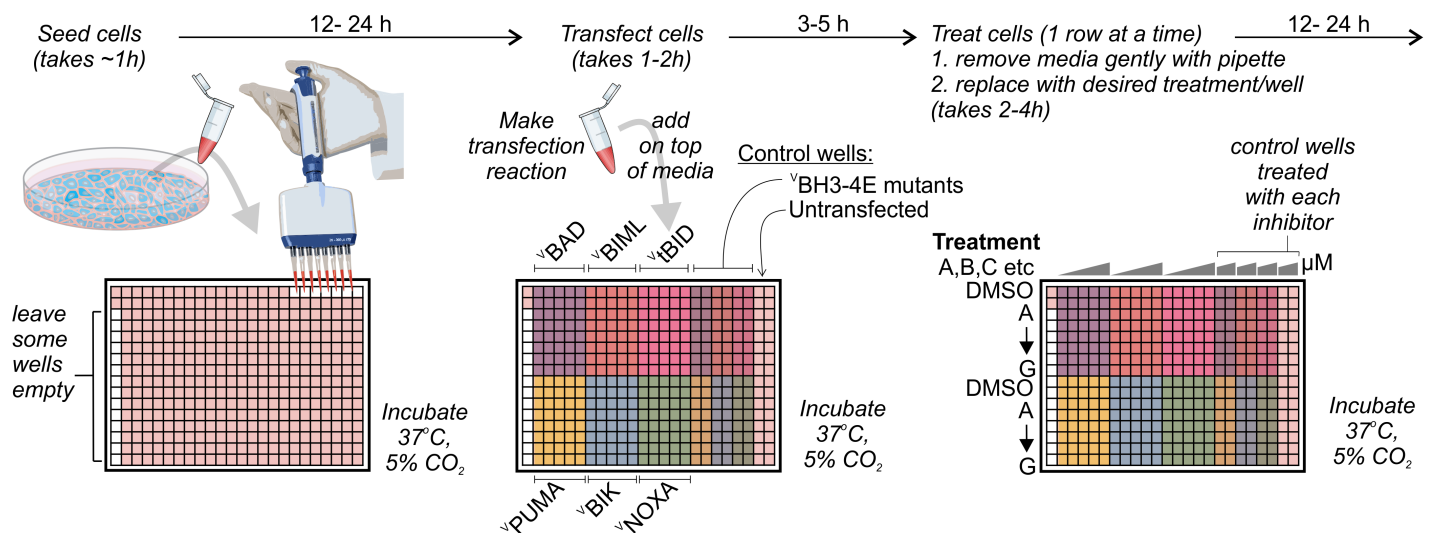
References

For our associated manuscript see Osterlund et al., 2022, "Efficacy and specificity of inhibitors of BCL-2 family protein interactions assessed by affinity measurements in live cells", Science Advances.

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Figures

A Prepare sample plate (3 days)



B Collect data on INO-FHS (1h setup + 24h automated imaging)

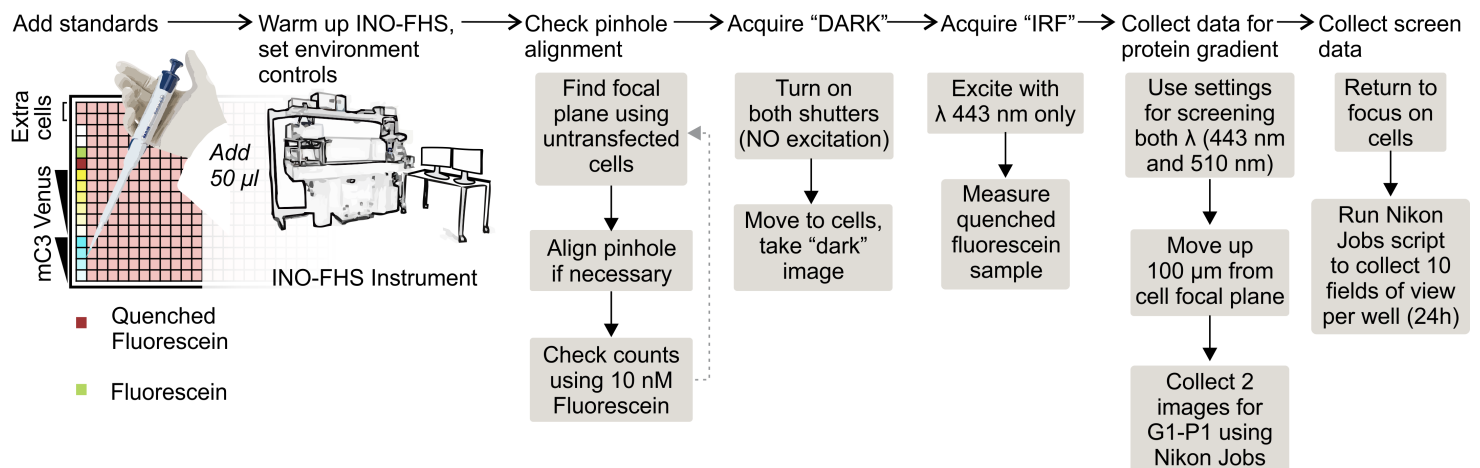
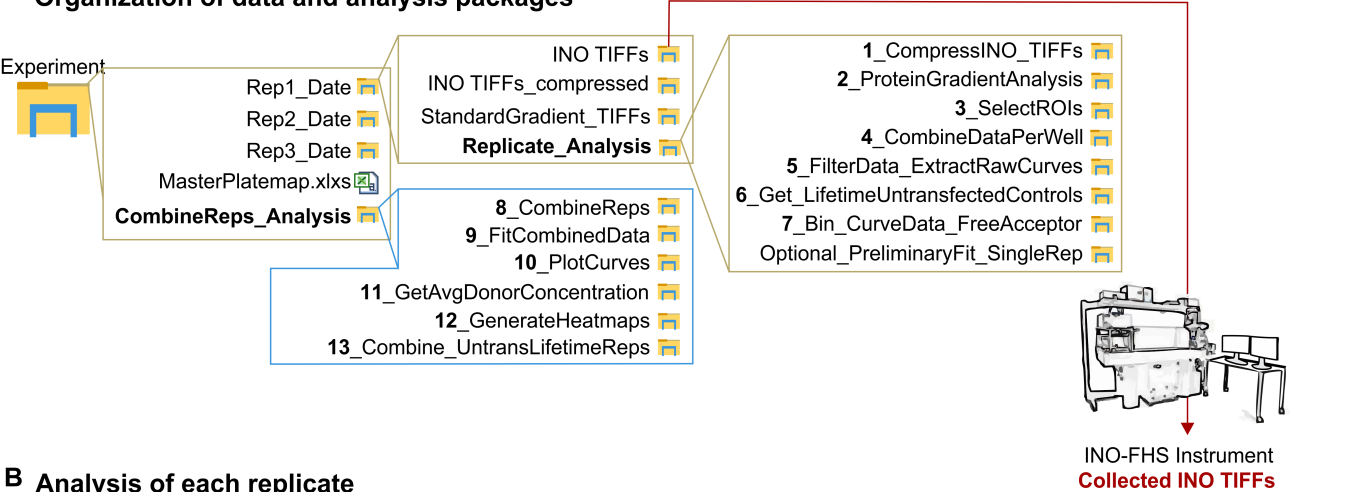


Figure 1

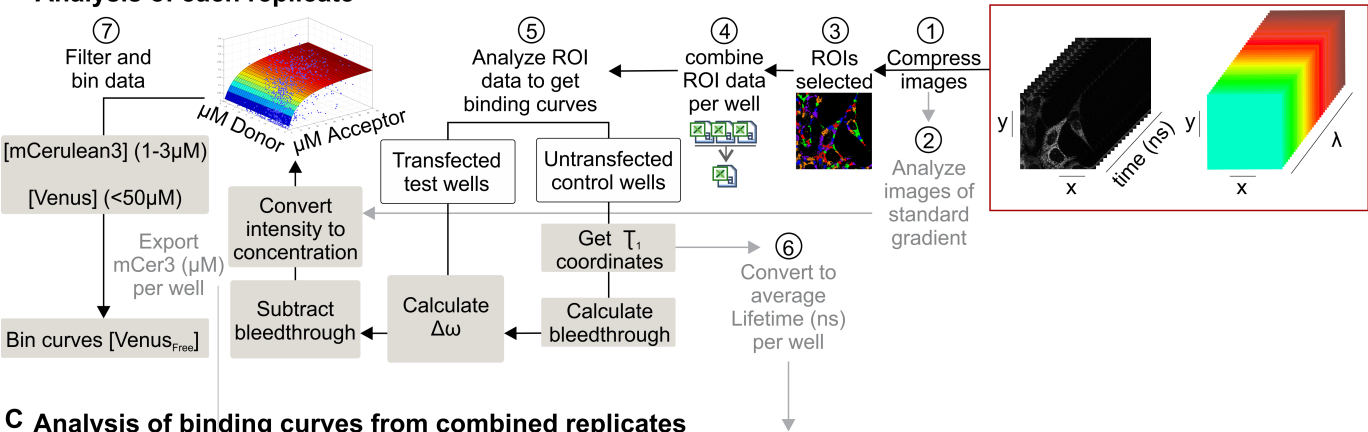
Sample preparation and data collection for quantitative Fast FLIM-FRET (qF3) (A) Example diagram of plate preparation with steps, necessary controls, and approximate time required in hours (h). Cells stably expressing mCerulean3-fusion (donor) were seeded on day 1, leaving space for fluorescent standards. Cells were then transfected with VBH3: Venus-fusion to BH3-only protein of interest (acceptor), where colours highlight the area transfected with each. Controls include 1) transfection reagent alone in "untransfected wells" 2) negative controls with a '4E mutation' made to the BH3 binding region. Before screening serial dilutions of each BH3 mimetic were made in anhydrous DMSO, to maintain the final 1:1000 dilution of DMSO in each well. 5h later we remove media row by row and replace with BH3 mimetic treatment: different drugs represented by 'A-G' per row, where concentration is indicated above. DMSO alone is used to treat untreated positive (test) and negative controls. VBH3-4E collisional controls and untransfected wells must be treated with at least the 2 highest doses to ensure no effect on donor lifetime. The plate is incubated ~12h before imaging. (B) Summary of data acquisition steps modified from our previous operation protocol⁶, to add required steps for qF3. Every experiment includes fluorescence standards of purified mCerulean3 and Venus protein of different concentrations, a 10nM

fluorescein, and quenched fluorescein (for IRF). In the plate, we also include an extra ‘disposable’ well of cells for focusing and aligning the pinhole.

A Organization of data and analysis packages



B Analysis of each replicate



C Analysis of binding curves from combined replicates

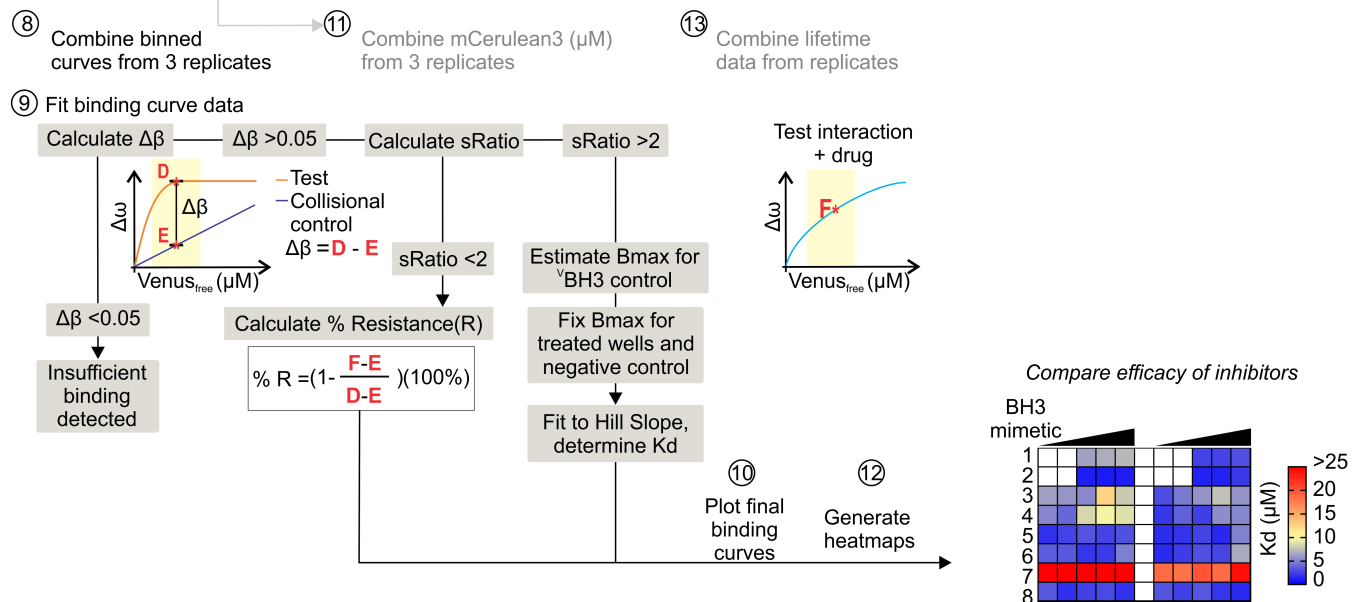


Figure 2

Summary of analysis steps in qF3 analysis pipeline (A) Data organization. “INO TIFFs” are original image data from INO FLIM Hyperspectral (FHS) instrument. The analysis folders (bold) are copied to each experiment, where subfolder names indicate the order of analysis steps. Following is a schematic of data

analysis, following black arrows starting at images collected on the INO-FHS, where steps 1-13 (circled) correspond to numbered folders in (A). Repeat the “Replicate_Analysis” (B) for three replicates then continue to the “CombineReps_Analysis” (C). (B) mCer3: mCerulean3; 1: unbound lifetime; [x]: concentration of ‘x’; Δ : fractional change in angular frequency: a value linearly related to the bound fraction of donor molecules. (C) Data analysis post combining replicates where the path splits indicate a decision to move forward in analysis Any Red asterisk represents the median Δ of points that fall between 10-20 μ M Venusfree (area in yellow) on each curve. Final steps 11 & 12 are used to generate all heatmap data for screen analysis, and 13 is used to examine the effect of each inhibitor on the mCerulean3 (donor) protein alone. In heatmaps, generally, we use blue for “binding” and “red” for non-binding and each box represents the analysis of a binding curve containing 3 independent biological replicates.

Automated ROI selection

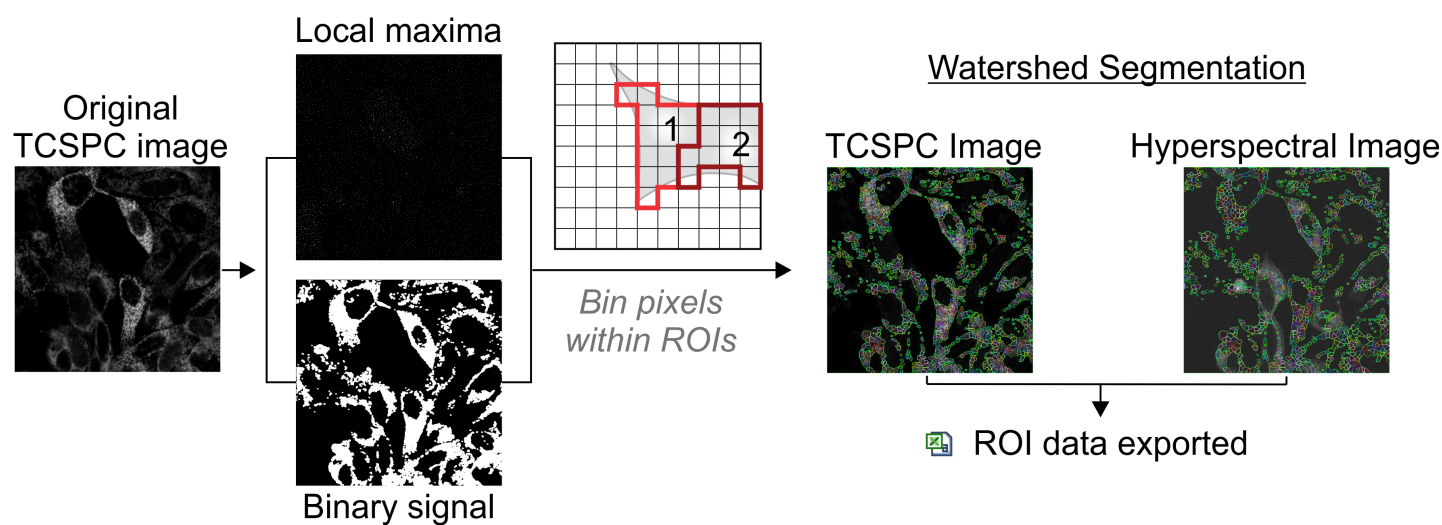
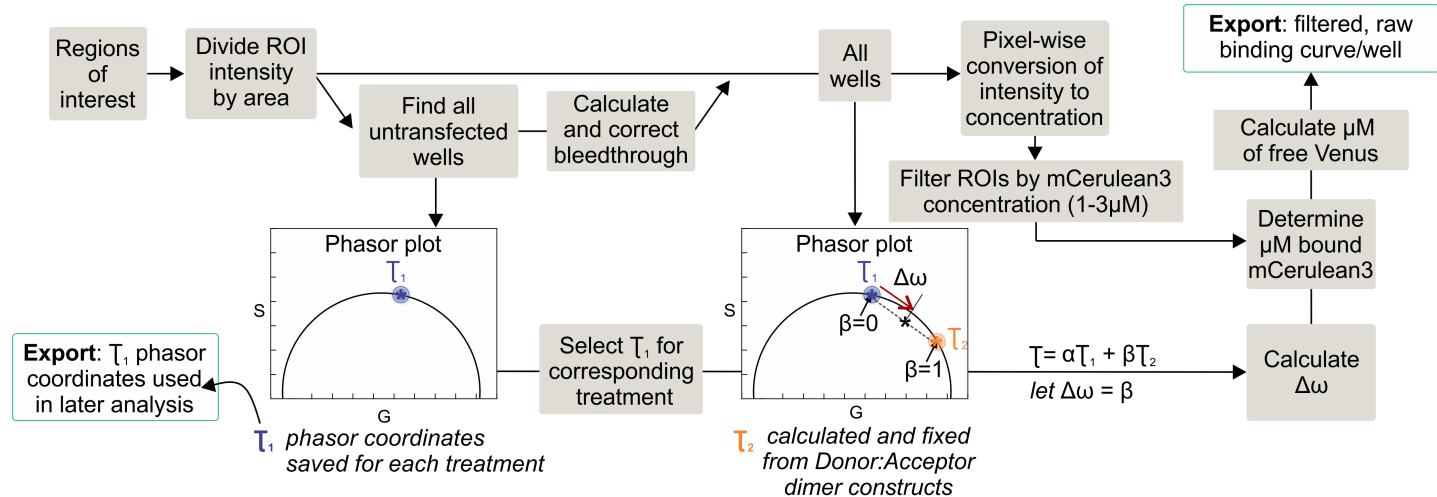


Figure 3

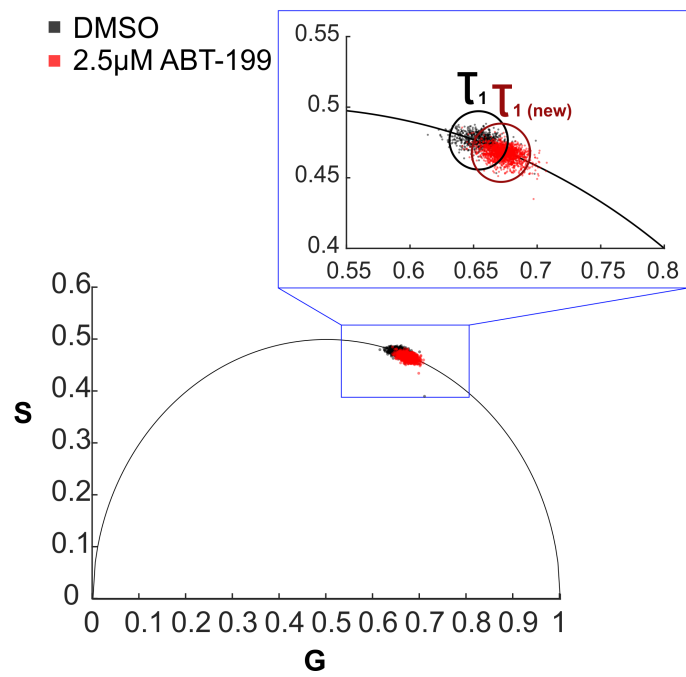
Example of the region of interest (ROI) selection. An example of a donor (mCerulean3 fused to BclXL) expressed in BMK-DKO cells is shown; collected using time-correlated single-photon counting (TCSPC) measurements, used in lifetime calculation. Two copies of the image are used to identify local maxima and pixels belonging to cellular structures. The first copy is dilated using a disk structural element. Pixels with equal value in the original image and the dilated image are identified as seed pixels (dots on local maxima image). The second copy is convoluted with a Laplacian of a Gaussian (LoG) filter and a mean threshold is used to generate the binary mask which identifies subcellular regions. Segmentation is achieved by deploying a Watershed Segmentation algorithm to fill in the regions between two identified seeds. The ROIs are shown to the right on the same image as well as applied to the corresponding images in the hyperspectral channel. The fluorescence lifetime decay and spectral profile for each ROI is constructed by binning the collected counts for pixels within each region. This reduces the computational burden as the number of analyzed profiles is equal to the number of ROIs and results in a higher signal-to-noise ratio associated with each analyzed profile. The fluorescence lifetime and spectral profile for all the regions of interest are saved to a comma-separated values (CSV) file for further analyses. CSV files

are combined to generate a single file per well then further processed to extract one binding curve per well.

A. Filter data and extract binding curves



B Check whether treatment affects donor lifetime



C Bin curve data

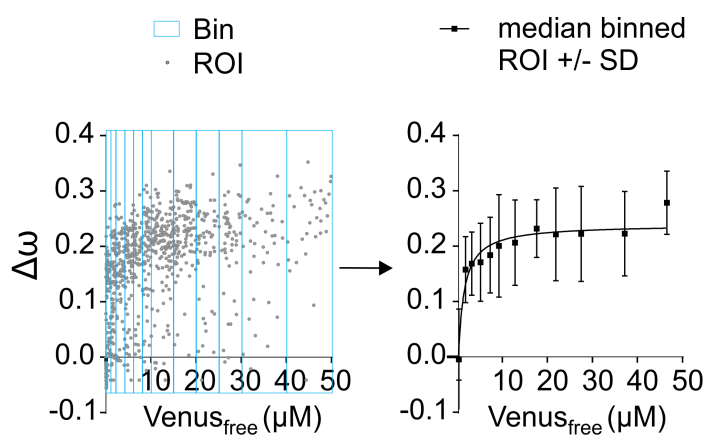
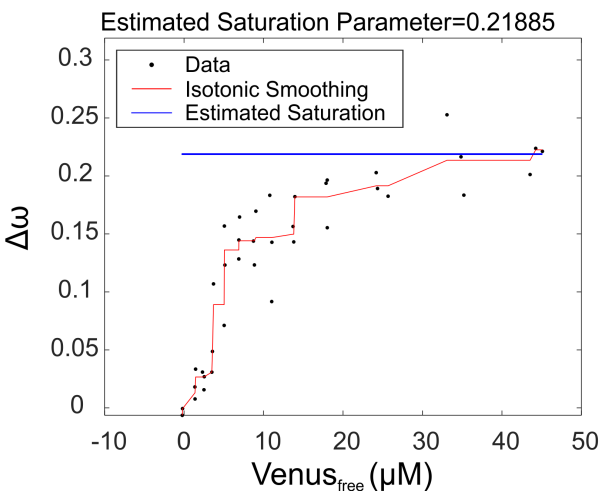


Figure 4

Replicate analysis (A) Details of steps in Analysis script Step 5, used to generate raw qF3 binding curves. Each screen plate contains control untransfected wells, treated with each drug to be tested. From combined .CSV files exported earlier, ROI decay data is plotted in phasor space. β : the bound fraction of donor molecules. In the absence of an acceptor, the unbound lifetime (τ_1) is shown in blue. Referred to as “bleedthrough” here is the intensity observed in the hyperspectral that is linearly related to counts in the TCSPC channel (mCerulean3). This is removed before calculating Venus concentration. τ_2 : the projected coordinates of the “bound” lifetime determined from extrapolating the fit of data collected from fluorescent dimers to intersect the universal circle. The τ_2 position was fixed in all analyses and τ_1 is determined for each drug treatment. For each ROI (e.g. * in phasor plot), we determine the fractional

change in angular frequency ($\Delta\omega$), or the distance from 1 to *, where $\Delta\omega = 1 - \beta$. $\Delta\omega$ is linearly related to the true bound fraction (β), but depends on FRET efficiency. In this script, we export phasor coordinates to examine the effect of each treatment on donor lifetime. The coordinates are determined by finding the center of mass of the distribution of ROIs. For example in (B) see phasor coordinates for ROIs from BMK-DKO cells expressing CBcl2, treated with DMSO (black) compared to treated cells (red). 1 new is the phasor coordinates of the unbound lifetime for all wells treated the same. (C) Example of exported raw qF3 binding curves (left), which are binned (see blue boxes) by the concentration of VenusFree to produce a binned binding curve per well.

A User feedback in fitting script (step 9)



B Result of simple curve plotting script (step 10)

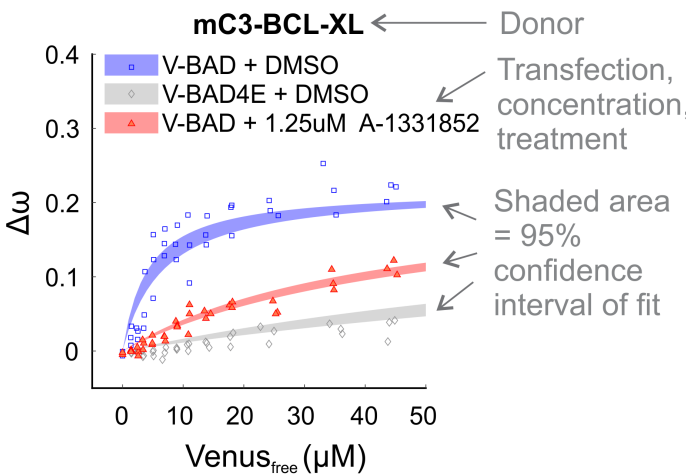


Figure 5

Plotting and fitting final qF3 data (A) Example pop-up window that appears in Analysis Step 9_FitCombinedData. For each positive control or 'test', the script will display the data along with an isotonic fit (Red) of that data. The script finds the median of the last 6 points in the curve to estimate the Saturation parameter (or Bmax) that will be used in fitting the Hill Slope equation (displayed above the graph). The user can override this step if desired, as described in the methods. The saturation parameter is also used as the maximum in defining the binding box that the dataset occupies. Then the script determines the area above and below the isotonic fit, which are used in calculating sRatio. (B) Example plot generated from Analysis Step 10 labelled to the right. On each curve, we plot the positive (blue) and negative (grey) controls for comparison to the test (red) (n=3). Data is fit to a Hill Slope, where Bmax is fixed based on the positive control (step 9) and hill slope =1. The same fitting parameters are applied to treated wells and the VBH3-4E negative control. The script only fits data that can get a Kd. ie. if sRatio <2 or $\Delta\beta < 0.05$, then only points will be displayed. Each graph is saved with the convention: Donor_Acceptor_Concentration_treatment.

Dissociation constant (Kd) (μ M VenusFree). Six acceptor- fusion proteins (VBH3) are listed at the top. For each VBH3 (positive control) and VBH3-4E (negative control) the top 2 rows of each heatmap are treated with DMSO only. BH3 mimetics are listed to the left of each row, with concentration above (μ M). These titles apply to all heatmaps (A-H).