Multiplexed Dark FRET Biosensors: *An accessible live-cell platform for target- and cell-specific monitoring of protein-protein interactions in 2D and 3D model systems*

Supplemental Information

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KEY WORDS

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Target-Specificity

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3D spheroid

Supplementary Information:

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METHODS

Molecular biology

Biosensor constructs were designed to facilitate subcloning of future genes of interest (GOI) using a standardized restriction enzyme mapping: 5'-Nhe1-[XFP1-BamH1]-GOI-[Kpn2I-XFP2]-Not1-3', where the inclusion of a 5' or 3' fusion XFP includes an additional restriction enzyme site. GOIs for this study included a 23 amino-acid 'GS' linker (GSGGGGSGGGGSGGGGSGGGGSG); alpha-Synuclein (NM_001375286.1; previously synthesized with removal of an internal BamH1 site), TNFR1 (NM_001065.4), and TNFR2 (NM_001066.3). XFP gene fragments for mNeongreen (mNg), mScarlet-I3 (mScI3), ShadowY (ShY), and ShadowR (ShR) constructs were synthesized and subcloned at GenScript (USA).

All biosensor plasmids constructs were transformed into NEB-5a E. Coli and DNA purified via PureLink™ HiPure maxiprep kit (ThermoFisher, USA), aliquoted and stored at -20ºC. All constructs were sanger sequenced confirmed prior to use in cell experiments (ACGT, Wheeling, IL). Supplemental Table ST1 provides a full detail of biosensors developed and tested in this study.

Cell culture

Cell cultures were maintained in an incubator with 5% CO_2 (Forma Series II Water Jacket CO_2 Incubator, Thermo Scientific) at 37℃. HEK293T (CRL-3216; ATCC, USA) were cultured in phenol red-free Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 2 mM GlutaMax (Invitrogen), heat-inactivated 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). SHSY5Y (CRL-2266; ATCC, USA) neuroblastoma cells were cultured in phenol red-free DMEM:F12 (Gibco) with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco), and non-essential amino acids (NEAA; Gibco). HMC3 (CRL-3304; ATCC, USA) immortalized human microglia were cultured in Eagle's minimum essential medium (EMEM; Quality Biological, USA), heat-inactivated 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco), NEAA, 2 mM GlutaMax, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate.

Plasmid Transfections

HEK293T, SHSY5Y, and HMC3 cells were passaged and transiently transfected with FLT-FRET biosensor constructs via Lipofectamine 3000 (Invitrogen, USA) for HEK293T cells (24hr transfection) and Lipofectamine 2000 (Invitrogen, USA) for SHSY5Y and HMC3 cells (24- to 72-hr transfection) according to manufacturer's instructions. Total transfected DNA was 2.0µg per well of a 6-well dish with donor construct never exceeding 0.5 µg DNA per-well using supplemental empty-backbone vector as additional DNA reagent. Expression of all biosensor constructs were confirmed by live cell fluorescence microscopy prior to FLT-FRET characterization (ImageXpress Pico; Molecular Devices, USA).

Neuro-Glial Spheroid Culture and Imaging:

Biosensor transfected SHSY5Y and HMC3 cells were passaged and transiently transfected as described above. The day after transfection, cells were passaged and resuspended in differentiation media 1 (complete DMEM:F12 media supplemented with 10 µM retinoic acid (RA)) as described previously[1]. Self-assembled neuro-glial spheroids were cultured in suspension using an orbital shaker in the incubator for 3 days. NGS were pooled and plated for imaging (black walled, glass-bottom 24-well plate) and FLT-FRET characterization (opaque bottom, black walled 384-well plates).

FLPR experiments, and fluorescence lifetime data analysis

Biosensor transfected cells were harvested from 6-well plates by incubating with TrypLE (Invitrogen, USA) for 4 min, quenched with complete medium, and washed in DPBS. Cells were diluted to 1.0E6 cells/ml and dispensed at 50 µl/well in black-walled 384-well plates. Neuro-glial spheroids were pooled and media exchanged in well with two complete volumes of PBS and plated into 384-well plates.

FLT was monitored using a fluorescence lifetime plate reader (Fluorescence Innovations, Inc) as described previously[2]. This FLT-plate reader has two laser lines, a 473nm laser for exciting mNg with a 517/20 bandpass emission filter and 532nm laser for mCh and mScI3 with a 620/10 bandpass filter. Data is collected sequentially in the plates from the same sample with laser attenuation adjusted via neutral density filters to scale signal intensity to the instrument response function. As described previously[2-13], time-resolved fluorescence waveforms for each sample/well were fit with single-exponential decays using least-squares minimization global analysis

software developed in Matlab to give donor-acceptor FLT (τ_{DA}) and donor-only FLT (τ_D). FRET efficiency (*E*) was then calculated based on Equation 1.

$$E = 1 - \left(\frac{\tau_{DA}}{\tau_D}\right) \; Eq.1$$

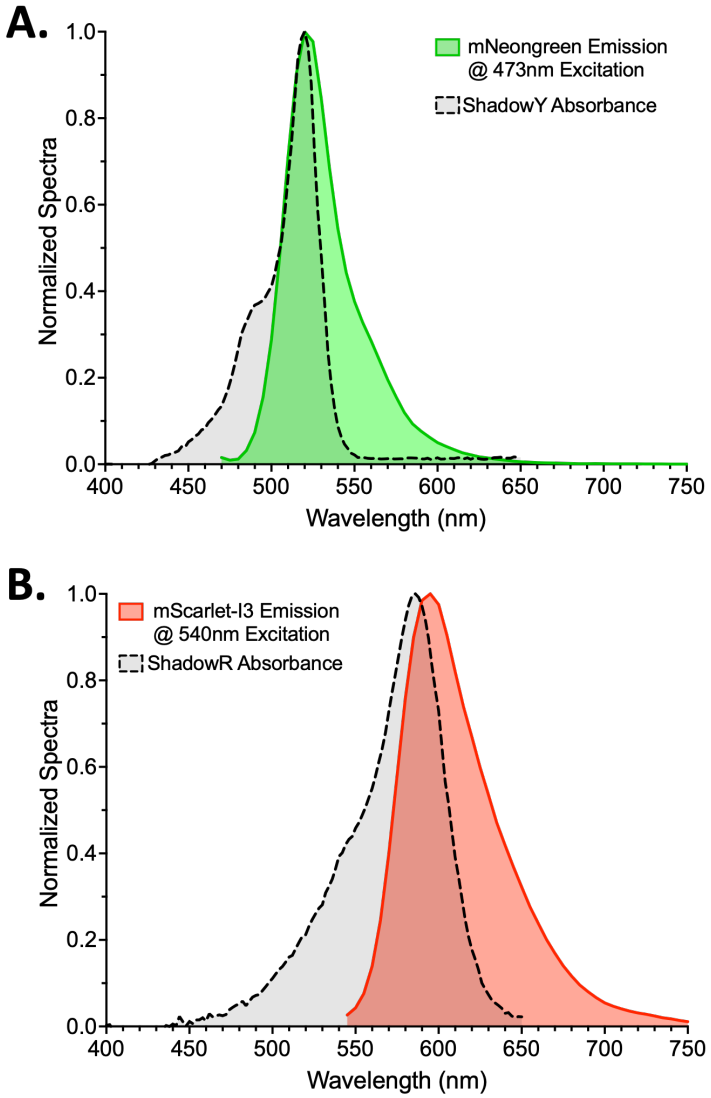
Statistical analysis

Data are shown as mean ± standard deviation unless stated otherwise. Statistical analysis for FLT and FRET experiments were conducted with one-way ANOVA using Prism v9.0 (GraphPad, USA) to determine statistical significance for all experiments. Values of p-value < 0.05 were considered statistically significant.

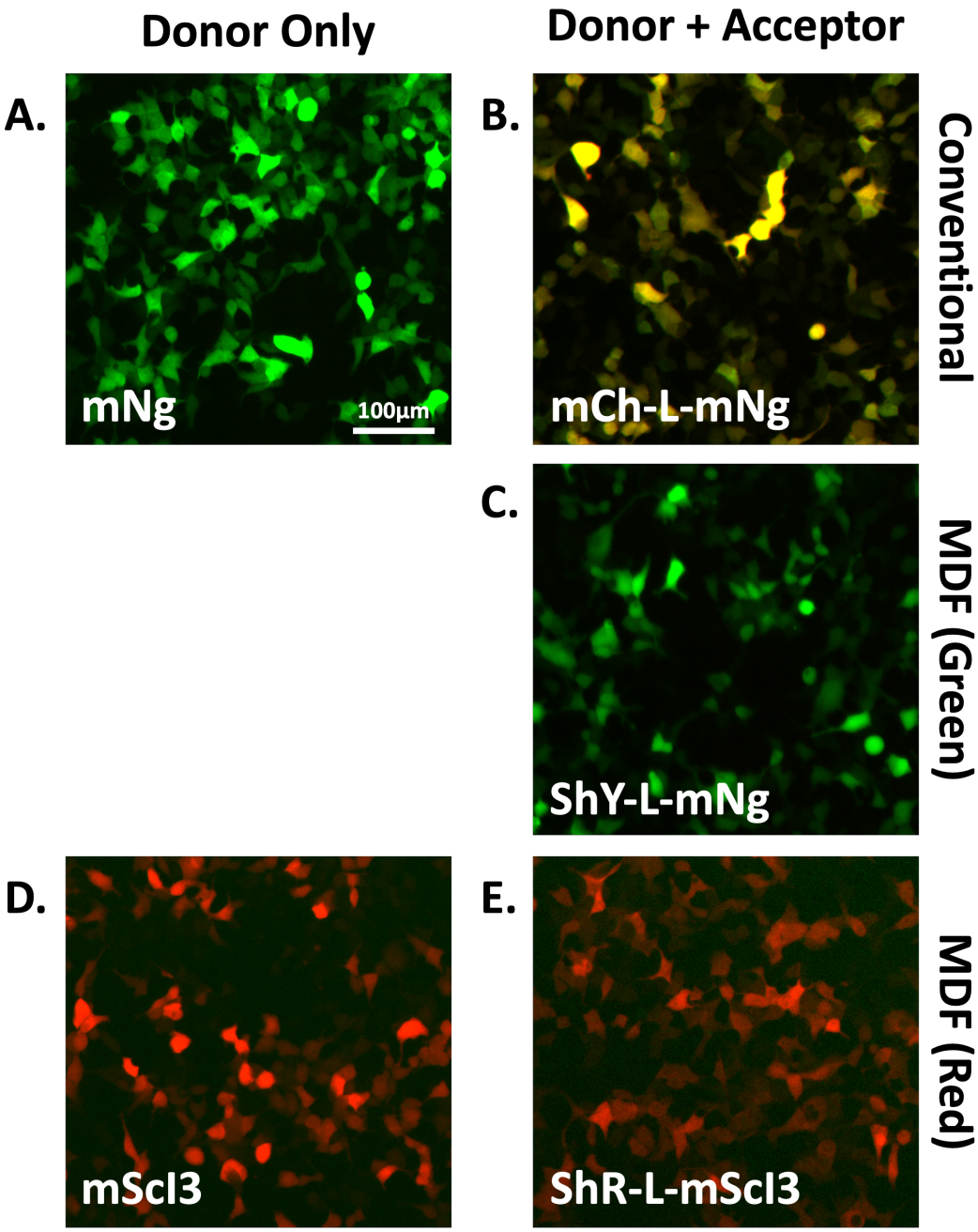
Supplemental Tables and Figures:

Supplemental Table ST1. List of all biosensor constructs developed for this study.

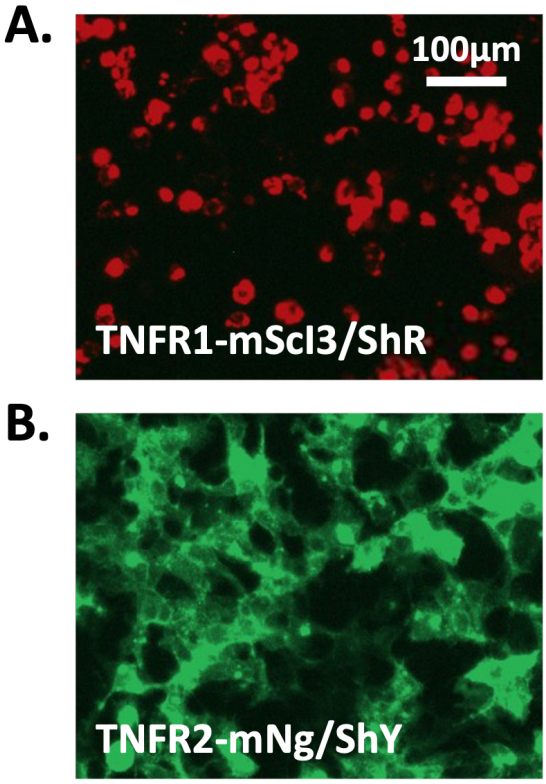
	Biosensors:	Role	Channel	Excitation/Absorbance Wavelength	Emission Filter
Control	mNeongreen (mNg)	Donor	Green	473nm	517/20
	mCherry (mCh)	Acceptor	Green	532nm	620/10
	mScarlet-I3 (mScI3)	Donor	Red	532nm	620/10
	ShadowY (ShY)	Acceptor	Green	N/A	N/A
	ShadowR (ShR)	Acceptor	Red	N/A	N/A
	mCh-Linker-mNg	FRET Positive Control	Green	473nm	517/20
	ShY-Linker-mNg	FRET Positive Control	Green	473nm	517/20
	ShR-Linker-mScI3	FRET Positive Control	Red	532nm	620/10
TNFSFR	TNFR2-mNg	Donor	Green	473nm	517/20
	TNFR2-ShY	Acceptor	Green	N/A	N/A
	TNFR1-mScI3	Donor	Red	532nm	620/10
	TNFR1-ShR	Acceptor	Red	N/A	N/A
aSyn	aSyn-mNg	Donor	Green	473nm	517/20
	ShY-aSyn-mNg	Donor + Acceptor	Green	N/A	N/A
	aSyn-mScI3	Donor	Red	532nm	620/10
	aSyn-ShR	Acceptor	Red	N/A	N/A



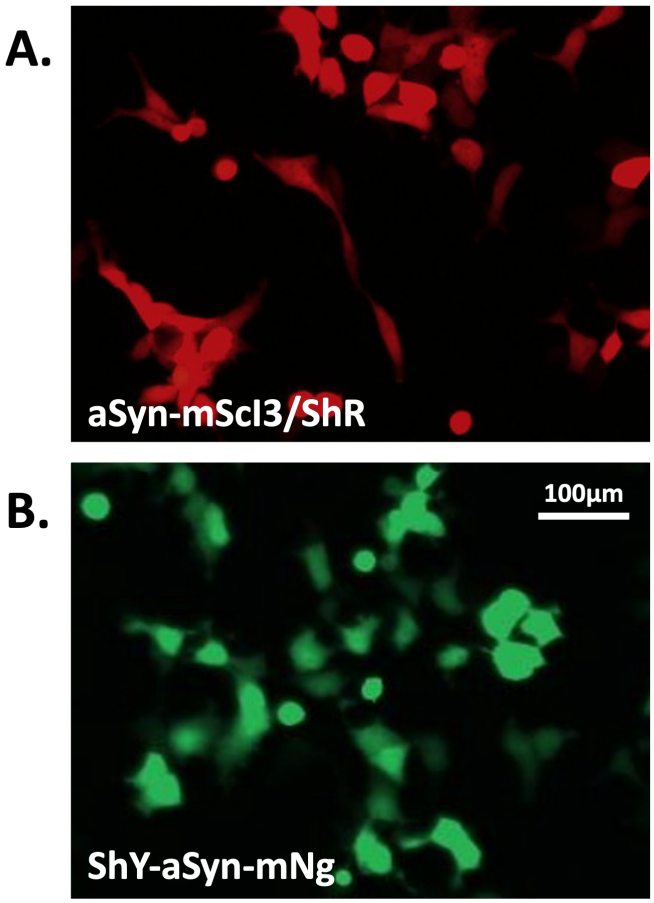
Supplemental Figure S1. Characterization of MDF FRET pair spectra. Emission spectrum for **(A)** donor mNg and absorbance spectrum for acceptor ShY; and **(B)** donor mScI3 and absorbance spectrum for acceptor ShR show large spectral overlap consistent of a robust FRET pair.



Supplemental Figure S2. Fluorescent micrographs for all control biosensors (donor only and donor + acceptor) were transiently transfected and expressed in HEK293T cells with **A.** mNg, **B.** mCh-L-mNg, **C.** ShY-L-mNg, **D.** mScI3, and **E.** ShR-L-mScI3. The Conventional FRET pair (mNg/mCh; **B**) is the only donor + acceptor condition with both green and red expression. Expression of control biosensors are diffuse, throughout the cell. Scale bar in **A** applies to all images.



Supplemental Figure S3. Fluorescent micrographs of transiently transfected HEK293T cells expressing **A.** TNFR1-mScI3/ShR and **B.** TNFR2-mNg/ShY biosensor. Expression patterns for both TNFR1 and TNFR2 show plasma membrane patterned localization. Scale bar in **A** applies to all images.



Supplemental Figure S4. Fluorescent micrographs of transient transfected aSyn single fusion (**A**) and double-fusion (**B**) biosensors. We have previously shown that diffuse expression of aSyn FLT-FRET biosensors reports on aSyn oligomerization[5,7]. Scale bar in **B** applies to all images.

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