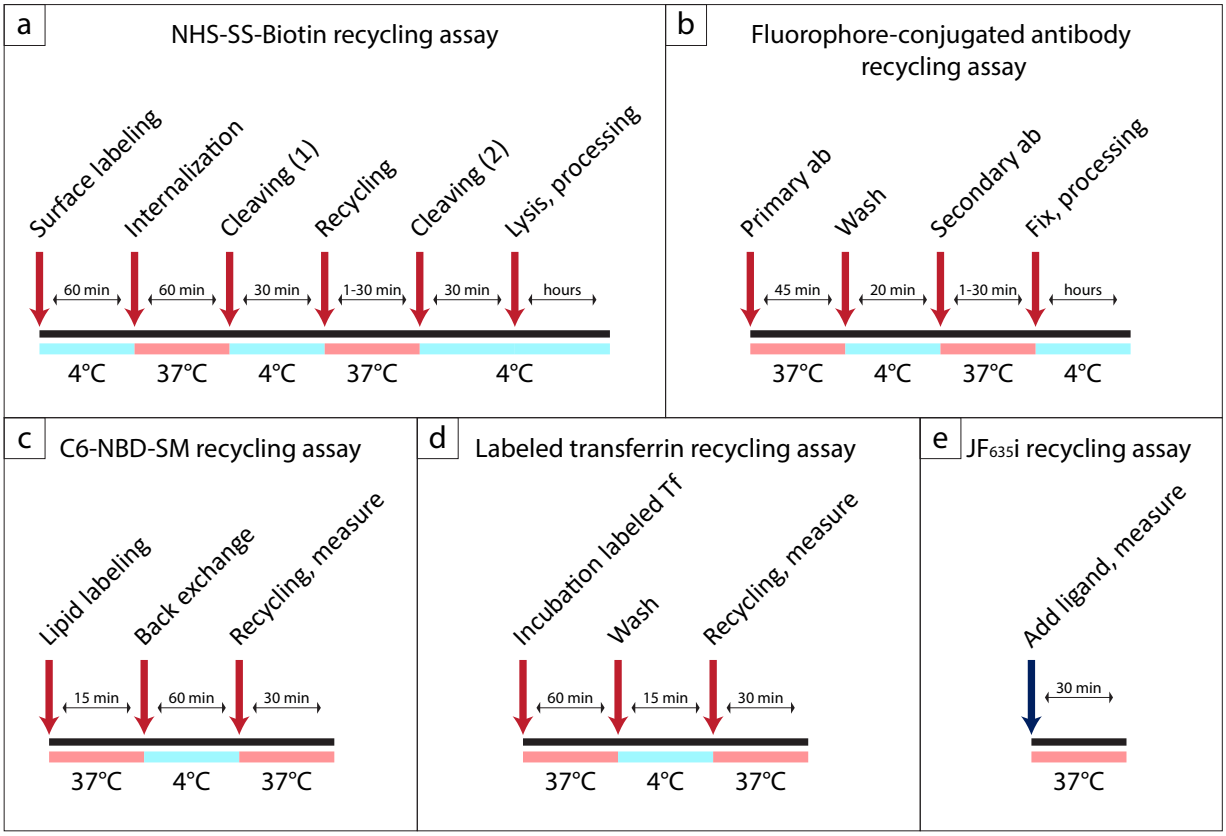
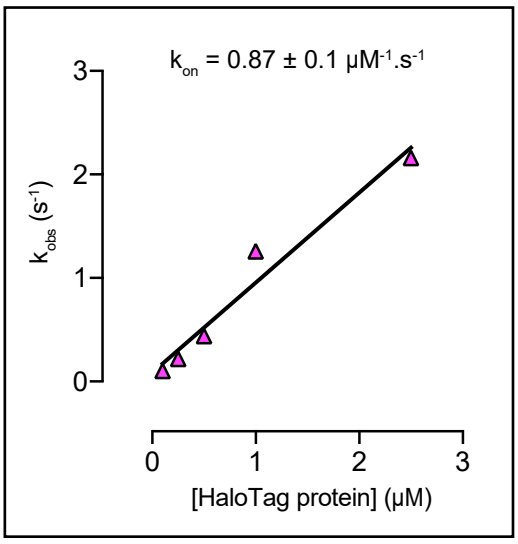
Figure S1 - Comparison of recycling assay protocols



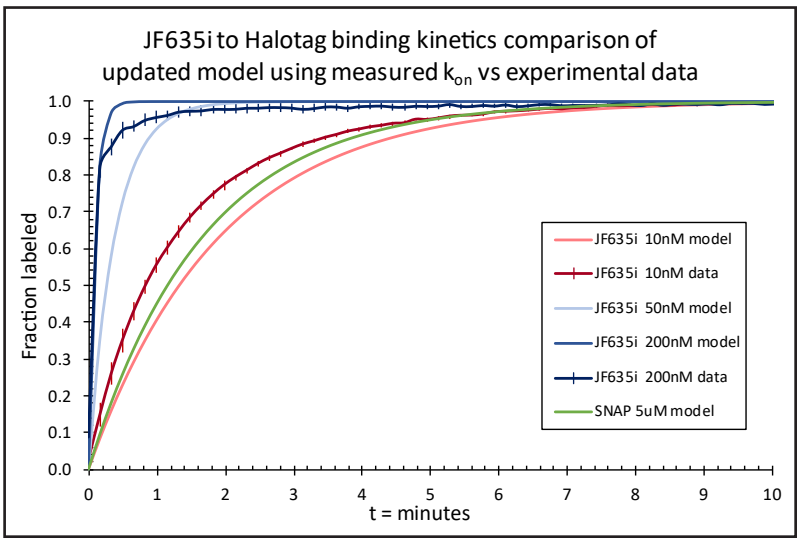
Schematic overview of the protocols of the different available recycling assays. a) First described in 2001[35], and still widely used today, in this assay cells are surface-labeled on ice using NHS-SS-Biotin. Cells are brought back to 37°C to allow internalization, put back on ice and surface label is cleaved. After cleaving, the cells are returned to 37°C for set timepoints to allow recycling. These samples are all processed by hand so the number of timepoints that can be collected is very limited. Other downsides are the continuous stopping and restarting of the endosomal system using cold washes and the requirement of an antibody suitable for ELISA or Western blot to the protein of interest. **b)** In the most recent version of this assay17, an antibody to the protein of interest or its genetic tag (e.g. HA or FLAG), is incubated with the cells at 37°C until it reaches steady-state. The cells are then washed on ice to remove unbound antibody, and the cells are returned to 37°C in medium containing a secondary antibody with a second color for a specific time. Although this method only requires a single temperature block, three additional disadvantages are that (i) one sample is needed for each timepoint, (ii) a suitable antibody is required, (iii) binding of antibodies to receptors at the cell surface can alter their internalization rate, thereby altering the recycling kinetics. **c)** First described in 198936, this assay uses a fluorescent analogue of sphingomyelin (C6-NBD-SM) to measure recycling. Although this method also has the disadvantage of requiring a pulse-chase and temperature block to generate an internal pool of labeled molecules, it has a clear advantage in that multiple timepoints can be taken from the same sample. A downside of this method is that it cannot be used to determine the exact kinetics of a single protein of interest, as it is lipid-based. **d)** Unlike any other receptor, recycled TfR returns to the plasma membrane together with its ligand. This unique behavior allows the implementation of an endocytic recycling assay based on labeling the ligand instead of the receptor. In its various reincarnations going back as far as 198321, cells are incubated with radiolabeled 125I-transferrin or fluorophore-conjugated transferrin at 37°C until steady-state is reached, washed on ice to remove unbound transferrin and returned to 37°C. Samples are then taken from the medium at specific times to quantify the radiolabeled or fluorescent transferrin released to the medium after endocytic recycling of the transferrin-TfR complex. Advantages of this assay are that many measurements can be made from the same sample and only a single low temperature block is required. A main disadvantage of this assay is that it is only applicable to TfR. **e)** Our proposed recycling assay requires no cooling or washing steps and therefore has a clear advantage over currently available recycling assays.

Figure S2 - Characteristics of JF635i HTL

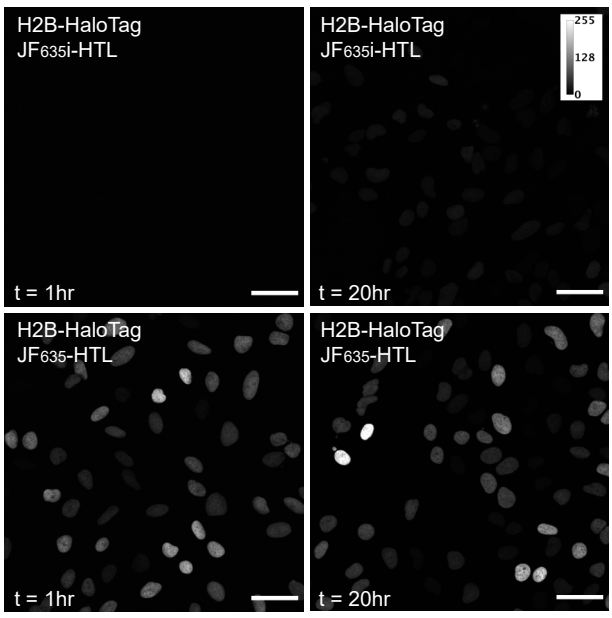
a



b

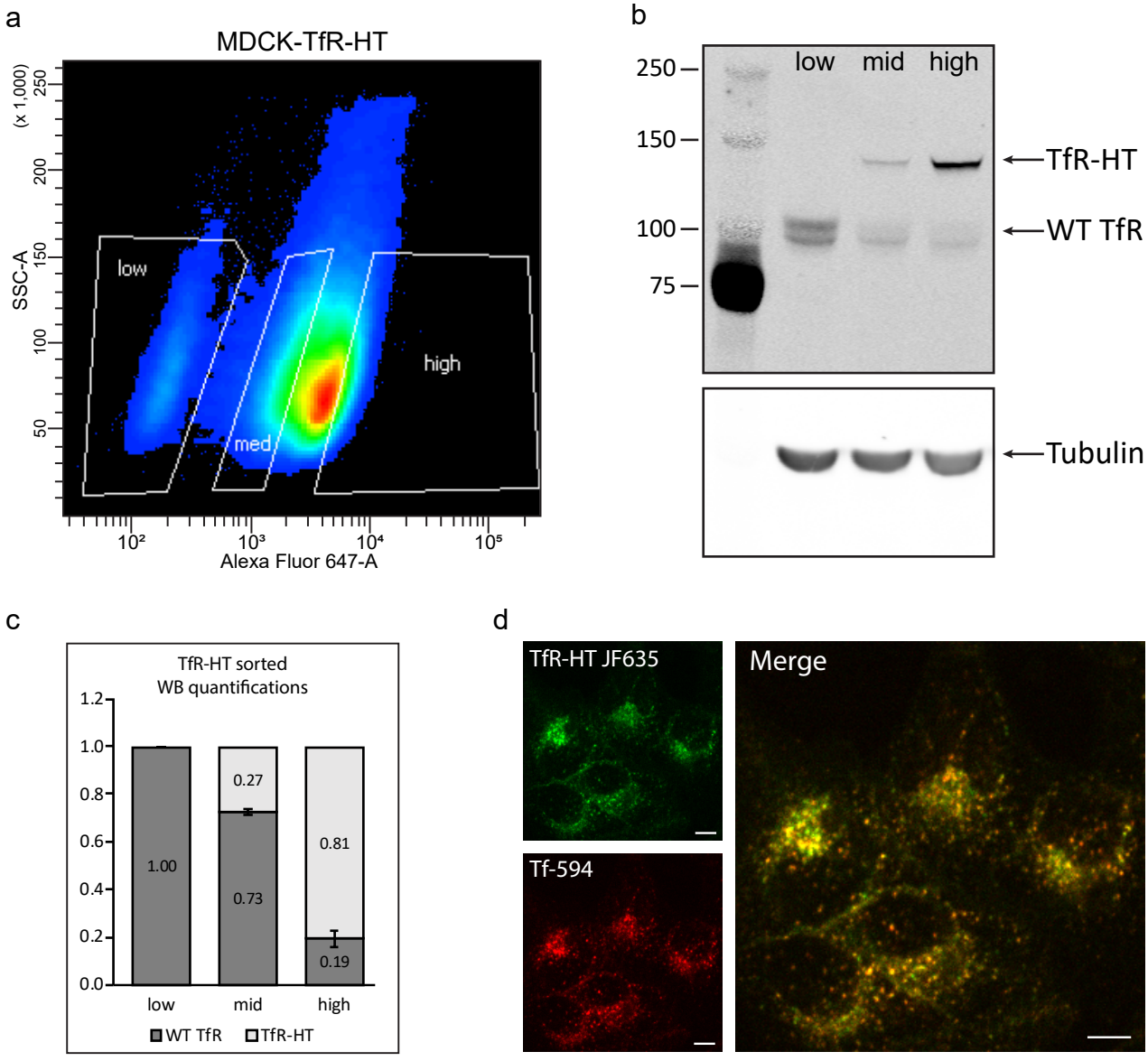


c



Characteristics of JF635i HTL a) Determining the kon of using stopped-flow analysis JF635i-HaloTag ligand results in a kon of 8.7x105 M-1s-1. Mean values of a triplicate experiment. **b)** The theoretical binding kinetics of HaloTag with different concentrations of JF635i-HaloTag ligand using the kon from the stopped-flow analysis (lines without error bars) compared to binding kinetics of JF635i-HaloTag ligand binding to a cell surface receptor in PFA-fixed cells (lines with error bars). Additional comparison with the current fastest non-permeable SNAP-tag ligand to date BGAN-2C4 (green line). Experimental datasets are n=4, error bars represent the standard deviation of the mean. **c)** Representative images of U2OS cells expressing H2B-HaloTag labeled with 200 nM JF635i- HaloTag ligand (top panels) or JF635-HaloTag ligand (bottom panels) for 1 h (left panels) or 20 h (right panels). All images are shown at the same intensity level; scale bars: 50 μm.

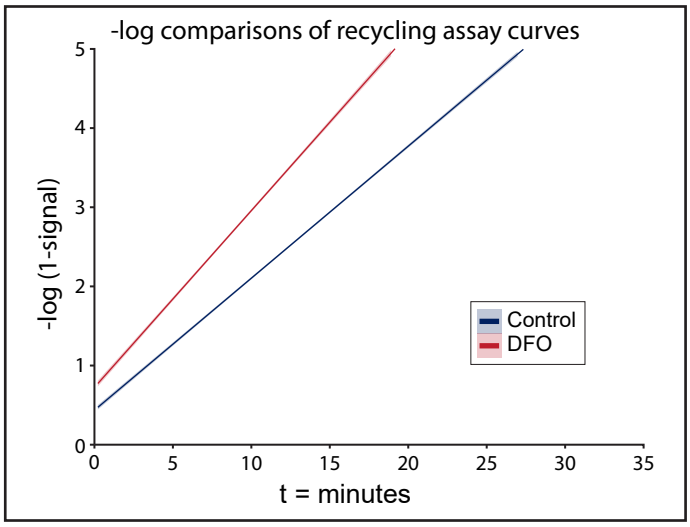
Figure S3 - Generation of a TfR-HT MDCK cell line



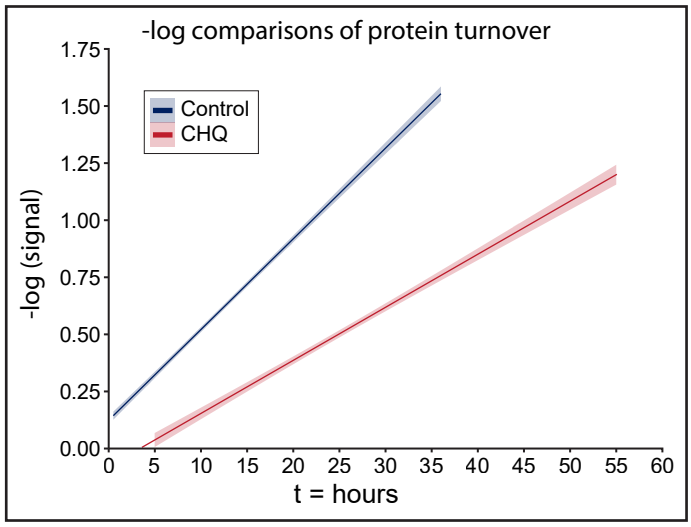
Generation of a TfR-HT MDCK cell line. a) Flow-cytometry plot of side-scatter versus 660 nm emission of MDCK TfR-HT cells labeled with permeable JF635-HaloTag ligand. The plot shows the gates used to isolate the low, mid and high TfR-HT expressing cell populations. **b)** Western blot analysis of lysates from low, mid and high expressing cells after flow cytometry sorting. Labeling with Mouse-anti-TfR shows progressively less wild-type TfR and more TfR-HT. **c)** Quantification of b). Triplicate experiment, error bars represent the standard deviation of the mean. **d)** TfR-HT cells were incubated for 30min with transferrin-594 (Tf-594) and for 1 hour with permeable JF635-HaloTag ligand before fixation. TfR-HT shows an almost complete overlap with Tf-594.

Figure S4 - Linear comparison of Recycling assay data

a



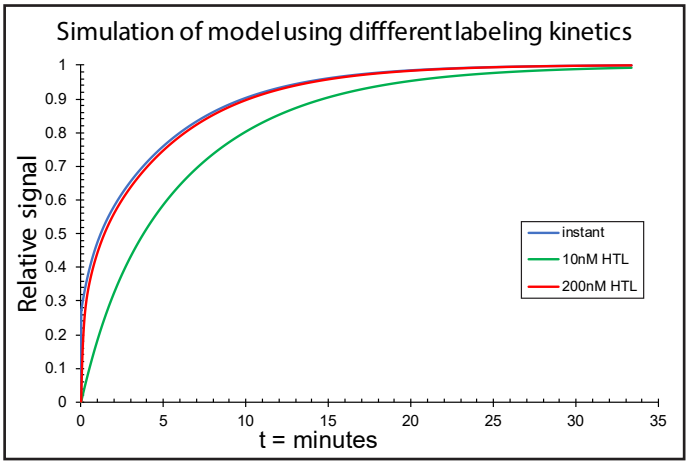
b



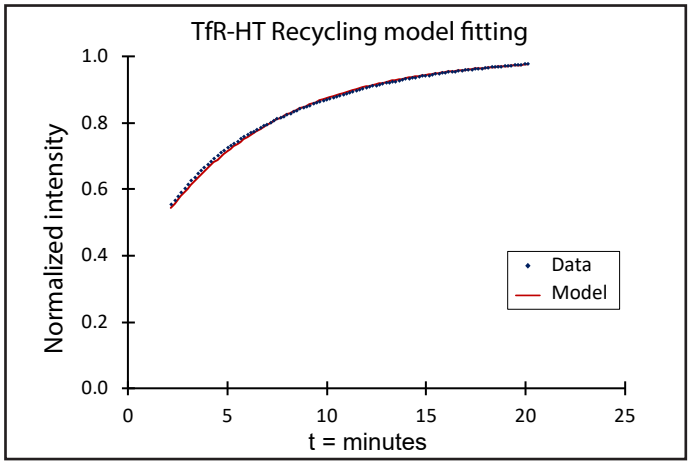
Linear comparison of recycling assay and protein turnover assay data. a) Graph depicting the linear fit over the log transformed recycling assay datasets (-log of 1 – the normalized signal). This representation allows accurate determination of the significance of the difference between datasets. The linear fit was performed over the dataset until maximum was reached. The light-colored band around the line represents the 95% confidence interval of linear fit with respect to the dataset. The linear fits have an adjusted R2 value of 0.897 and 0.761 for control and DFO respectively. The difference between control and DFO was significant with a p-value of <1x10-5. **b)** Graph depicting the linear range in the log transformed protein turnover datasets (-log of the normalized signal). This representation allows the accurate calculation of the protein-half life and significance of the difference between datasets. The light-colored band around the line represents the 95% confidence interval of linear fit with respect to the dataset. The linear fits have an adjusted R2 value of 0.947 and 0.732 for control and CHQ respectively. The difference between control and CHQ was significant with a p-value of <0.001.

Figure S5 - Fitting of Recycling assay data to the model

a



b

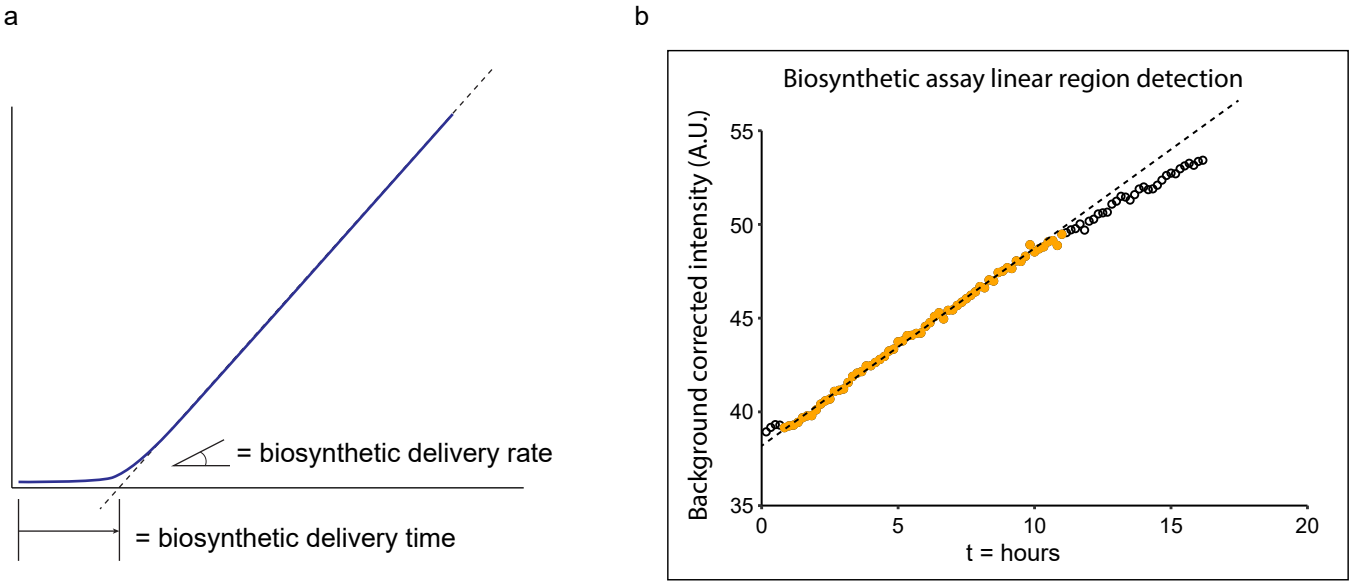


c

k_{01}	0.0092 (sec^{-1})
k_{10}	0.0071 (sec^{-1})
k_{12}	0.0055 (sec^{-1})
k_{20}	0.0026 (sec^{-1})
X_0	0.31
X_1	0.22
X_2	0.47

Fitting of the recycling assay data to the model. a) A kinetic model of the recycling assay that uses a separate kinetic rate constant for receptor labeling at 10nM (green curve) and 200nM (red curve) HaloTag ligand concentration compared to a kinetic model that assumes instant labeling of the receptor (blue curve). This resulting graph shows near-identical curves for 200nM and instant labeling. **b)** Fitting of the mean values from the recycling assay to the kinetic model. The model was fitted over the data between 2 and 20 minutes. **c)** Table with the rate constants (k) and relative occupancy (X) values of the recycling assay control dataset calculated using the kinetic model.

Figure S6 - Linear fitting of biosynthetic assay



Linear fitting of the biosynthetic assay. a) Schematic representation of the two kinetic measurements that can be obtained from the biosynthetic delivery assay. The slope of the linear part of the curve represents the biosynthetic delivery rate, while the time between t=0 and the start of the linear curve represents the time needed for newly synthesized proteins to reach the plasma membrane. **b)** Unbiased determination of the linear range allows a linear fit of the data and establishing the intersection with starting value to calculate the biosynthetic delivery time.

Movie 1.
Representative acquisition of Control cells and DFO-treated cells at one frame per 10 seconds for 45 minutes. Rate of signal accrual over time is markedly different between the two conditions. Quantifications are presented in figure 3.