

Supplementary Material for

Flow Radiocytometry Using Droplet Microfluidics

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Abstract

The supplementary material summarizes nomenclature and provides notes and figures to further describe the experiments and the results.

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List of Nomenclature

W	channel width [m]
H	channel height or depth [m]
R_{mean}	average positron range [m]
R_{max}	maximum positron range [m]
S	signal: peak height of drop fluorescence

B	background: averaged valley height of continuous phase fluorescence
I	fluorescence intensity of irradiated sample
I_0	fluorescence intensity of nonirradiated sample
Φ	sensitivity of ROS probe [Gy ⁻¹]
D	radiation dose [Gy]
Φ_{4h}	sensitivity of ROS probe measured 4 hours after irradiation [Gy ⁻¹]
$\Delta\Phi\%_{4h}$	relative loss of sensitivity after 4 hours
L	average spacing between single-cell containing drops [m]
a	drop diameter [m]
d	distance between optical fiber end and drop read channel [m]
l	center-to-center drop spacing [m]
k	number of particles in a drop
$\bar{u}$	average velocity of channel flow [m/s]

Greek Symbols

α	ratio of radioactivity to DCFH concentration in sample [GBq/nmole]
φ	single cell-encapsulation efficiency
ψ	throughput in drop read [s ⁻¹]
λ	average number of particles per drop volume

A. Incubation system

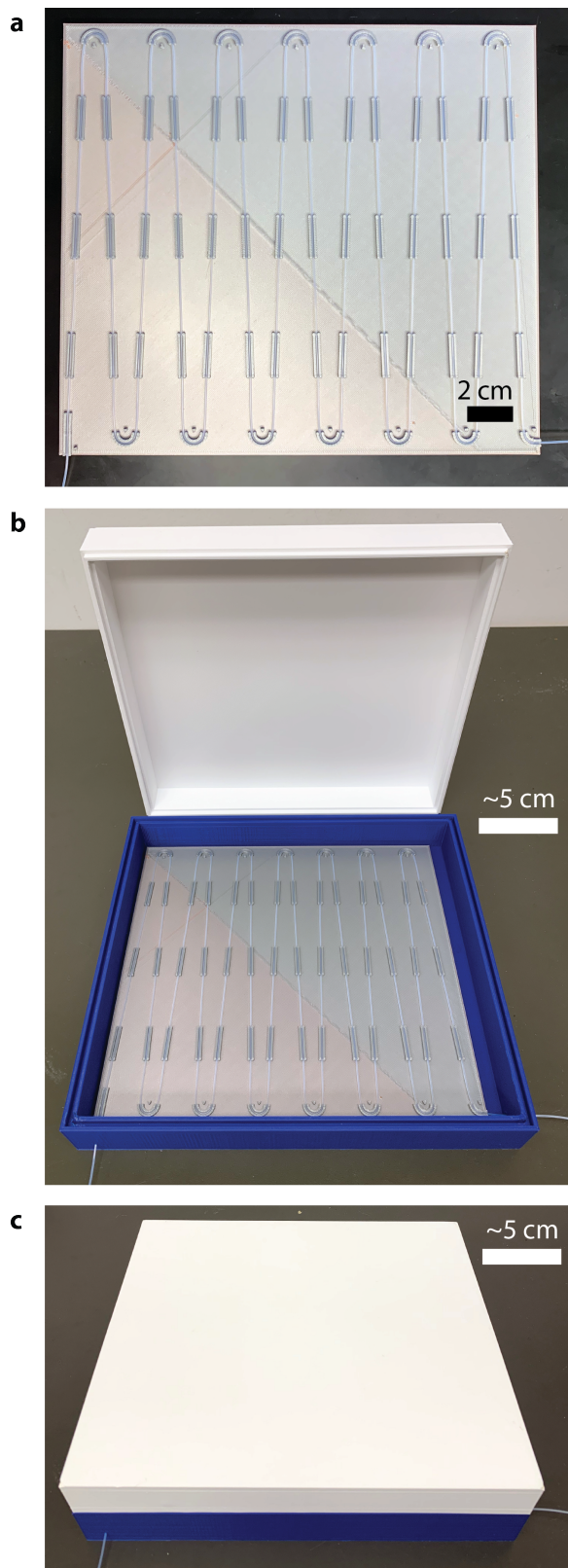


Figure S1. Incubation tubing and its holder and box container. (a) Top-view of the tubing holder (b) Open box containing the tubing and holder (c) Closed box

It was important to keep cells near the center of their respective drops during incubation. Due to density mismatch, cells sank down within a drop and remained at the bottom of the drop sphere. This is undesirable as almost half the positrons emitted from the sunken cell likely escape from the cell-containing drop and either generate no useful signal or, worse, cause unwanted radiofluorogenic conversion in adjacent drops. To address the issue, we continuously rolled the drops to stir their contents and prevent the cells from being stuck at the bottom. This procedure significantly enhanced radiofluorogenic efficiency of the cell-containing drops. Having a tubing end connected to an oil-filled syringe and the other to the inlet port of a drop read microchip, we programmed a syringe pump to keep moving the fluid back and forth throughout the incubation period.

The tubing was laid out flat horizontally with the aid of a 3D-printed holder as shown in Fig. 2e and Fig. S1a. The holder served to keep the tubing incubator leveled and prevent drops from buoyancy-driven packing, evenly distributing the drops throughout the long tubing during incubation. This helped maintain desired spacings between drops and minimize unwanted cross-contamination between radiolabeled cell-containing drops. In addition, the tubing and holder were placed in a light-tight box to prevent unwanted light-activated conversion of DCFH to DCF (Figs. S1b and S1c). We put ice over the tubing inside the box to keep drops cold. This was, first, to suppress cellular production of ROS by lowering metabolism. Second, we aimed to decrease lateral diffusivity of ROS by tightly packing cell membrane lipids and minimize unwanted leakage of inherent ROS into drops.

B. Detection of peaks and valleys and measurement of its heights from drop read data

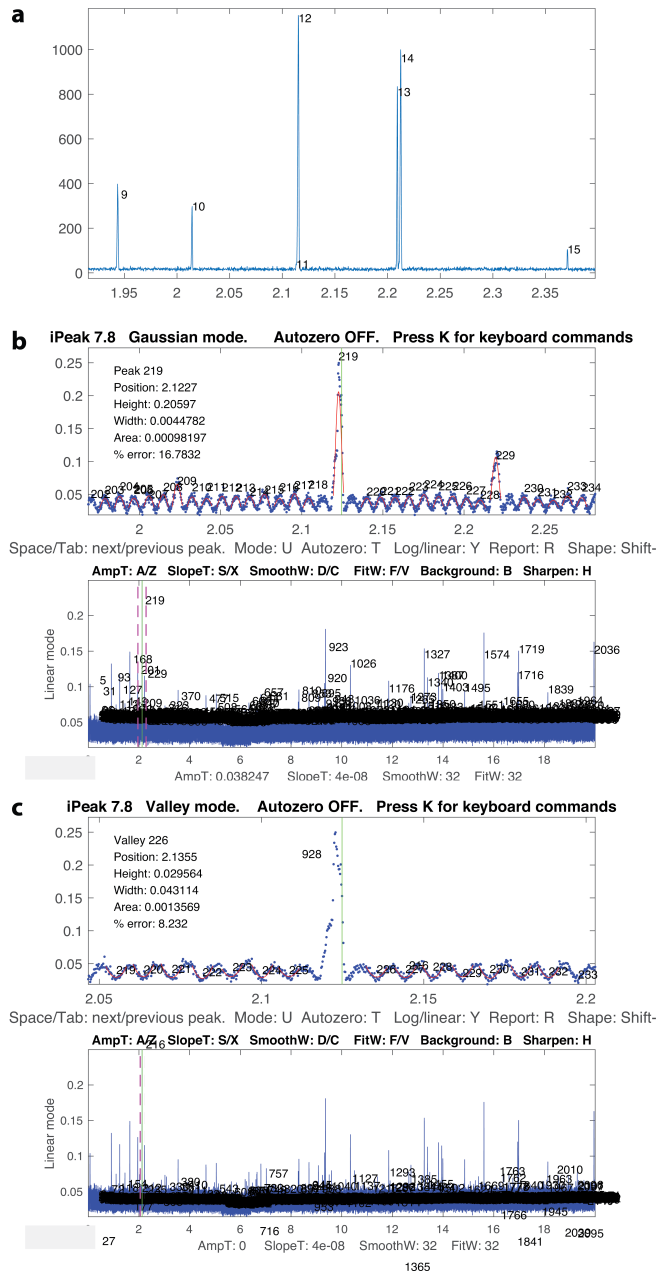


Figure S2. Snapshots of MATLAB codes-based detection of peaks and valleys and measurement of its heights and locations from PMT data. (a) Measurement of peak heights and locations from quantum dot fluorescence measurements. (b) Measurement of peak heights and locations from drop fluorescence measurements. (c) Measurement of valley heights and locations from drop fluorescence measurements.

Using a peak detection MATLAB code freely shared by Prof. Tom O'Haver (<https://terpconnect.umd.edu/%7Etoh/spectrum/>), peaks and valleys were detected and measured of its heights and locations from PMT data. First, peak heights and locations were measured from quantum dot fluorescence measurements (Fig. S2a). Second, heights and locations of peaks and valleys were measured from DCFH drop fluorescence measurements (Figs. S2b and S2c). As there were some broken-up drops and erroneous detection of peaks, we manually sorted out the wrong measurements and excluded those from our data after running the codes. Matching the first and second peaks in location (i.e., elapsed time), we differentiated fluorescence measurements of single cell-encapsulating drops from those of empty and multiple cell-encapsulating drops. We created a home-made MATLAB code to facilitate the matching process.

C. Cell encapsulation efficiency

Given cellular volume fraction is small (i.e., cells are sparsely distributed), studies have shown that cell encapsulation with a randomly distributed feedstock agree well with Poisson statistics. The Poisson distribution is given by,

$$p(k, \lambda) = \frac{\lambda^k e^{-\lambda}}{k!},$$

where k is the number of particles in a drop and λ is the average number of particles per drop volume. Tables S1 and S2 compare the measured cell encapsulation efficiencies to the predicted based on Poisson distribution. From the drop read data, λ were measured respectively as 0.041 for the shGLUT1 assay and 0.033 for the shSCR assay.

Table S1. Encapsulation efficiency of shGLUT1

k	0	1	2	3
$p(k, \lambda)$ (%)	96	3.9	0.081	0.001
Measured (%)	96	4.0	0.068	0.000

Table S2. Encapsulation efficiency of shSCR

k	0	1	2	3
$p(k, \lambda)$ (%)	97	3.2	0.053	0.001
Measured (%)	97	3.2	0.093	0.009

D. Design of channel and drop dimensions

We aimed to design an optofluidic microchip such that the inserted optical fibers could collect quantum dot fluorescence emission from a cell within a drop, in addition from the fluorescence of the drop itself. This is important to accurately count the number of cells contained in a drop. For efficient collection of the emissions, we used optical fibers with the largest numerical aperture and core diameter available to purchase, respectively 0.22 and 105 μm . In addition, we minimized the distance between the optical fiber ends and the fluid channel ($d = 50 \mu\text{m}$ as shown in Fig. 3a) to achieve the required coverage. This also helped collect the most fluorescence emission from radiofluorogenic drops. Based on this design, the circular field of view at a plane distanced 100 μm away from each optical fiber was approximately 120 μm in diameter. This puts a constraint on drop spacing. In order to read drops one by one with no crosstalk, in other words to have only one drop resident within the field of view along the streamwise direction, the center-to-center spacing between drops, l , needs to be at least 120 μm . Throughput in drop read is inversely proportional to the spacing. We are interested in the average spacing between single cell-containing drops, L , which is calculated as $L = l/\varphi$, where φ is the single cell-encapsulation efficiency. Throughput is then described as $\psi = \bar{u}/L = \bar{u}\varphi/l$, where $\bar{u}$ is average flow speed in the fluid channel. The throughput in drop read can be increased as we increase the flow speed. Yet the

flow speed should be limited by factors influencing measurement accuracy such as PMT sensitivity.

With these constraints in mind, we aimed to find the optimal diameter of the drops to maximize both radiometric accuracy and throughput. There are two more given conditions to take into consideration: the positron range of ^{18}F (i.e., $R_{\text{mean}} = 0.6 \text{ mm}$) and the typical radioactivity of a radiolabeled cell (i.e., 10 – 30 Bq per cell on average). A possible drop diameter, a , would range from a slightly larger than the diameter of a cell (i.e., $\sim 12 \mu\text{m}$) to the maximum positron range (i.e., $R_{\text{max}} = 2.4 \text{ mm}$), practically between 30 μm – 300 μm . To alleviate the cross-contamination issue during incubation, we aimed to generate 5 – 20 times more empty drops than occupied drops to space out the cell-containing drops. This corresponds to an encapsulation efficiency of 0.05 – 0.2. Such a low encapsulation efficiency is also useful for maximizing the desired the rate of single-cell encapsulation relative to multi-encapsulation as predicted by Poisson distribution. For our incubation system, relatively larger drop diameter was desired to have such spacing and encapsulation efficiency. If the drop diameter is relatively too small, then significantly larger number of empty drops are required to create spacings between cell-containing drops. This requires a significant lower encapsulation efficiency, which lead to a much lower throughput in drop read. Assuming drops are arranged in a planar 2D fashion during incubation, $\varphi \propto a^{-2}$, thus $\psi \propto a^{-2}$.

Drops shall not be too big. Considering that there is an optimal DCFH concentration relative to a given radioactivity and drop volume, use of a large drop would require DCFH concentration to be equally low. Due to the limit of our PMT sensitivity, 1 μM or higher DCFH concentration was required to achieve high SBR (i.e., ratio of specific to non-specific radiofluorogenesis). This was

important to accurately assess a wide range of cellular heterogeneity in metabolism. In the end, after testing 40 μm and 150 μm drop diameters in preliminary experiments, we settled on 110 μm drop diameter and 5 μM of DCFH concentration in our drop samples.

Given the desired drop diameter, we designed the channel depth and width to be 100 μm and 50 μm , respectively. Plug flow of drops was desired for uniform measurement conditions, and the cross-sectional area of the fluid channel was required to be smaller than that of the spherical drops. The aspect ratio of channel depth to width was limited to 2:1, otherwise PDMS is prone to tearing when peeled off from its mold.

We used two-photon polymerization 3D printing (Photonic Professional GT2, Nanoscribe GmbH, Germany) in the multi-depth mold fabrication. To our knowledge, this represents the first demonstration of 3D printing for fabricating the mold of entire microchannels including inlets and outlets. The 3D mold printing enabled an easy and independent shaping of each microfluidic component, optimizing its respective functionality. An example is shown in Fig. 2f. The inlet channel of DropRead microchip had an optimal depth of 40 μm at the inlet to efficiently filter out contaminants larger than cells, and the depth increased to the ideal 100 μm for drop read. the chip includes a ramp to smoothly connect the two channels of different depths, thus preventing bubble trap or drop-let breakup.

E. Reevaluation of DCFH stability and sensitivity

The protocol Ha et al. employed to prepare DCFH from DCFH-DA is as follows:

- (1) Dissolve 0.025 g of DCFH-DA into 5 mL of methanol (10 mM DCFH-DA).

- (2) Add 625 μL of this 10 mM DCFH-DA solution to 2.5 mL of 0.01 N NaOH contained in a 15 mL Falcon tube.
- (3) Keep it at dark and room temperature for 30 min.
- (4) Add 12.5 mL of pH 7.4 5X PBS to the DCFH-DA/methanol/NaOH solution (final DCFH-DA 400 μM).

The product was used to obtain the results shown in Fig. S3. Fluorescence change of the DCFH samples after X-ray irradiation was measured in time using kinetic spectroscopy. The sensitivity and the stability of DCFH to X-ray were calculated from linear regression based on the measured data.

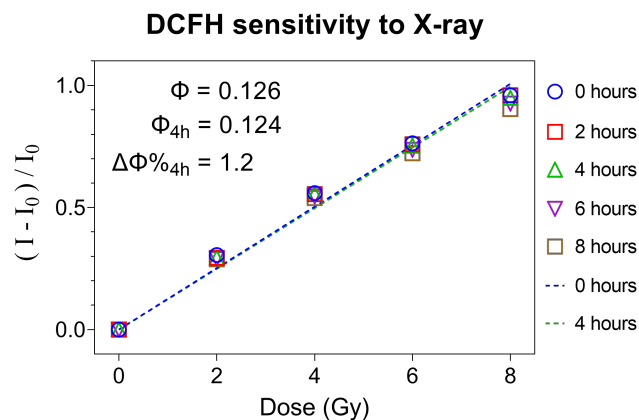


Figure S3. Reevaluation of DCFH sensitivity and stability.

F. Investigation of nonspecific activation of DCFH fluorescence

The fluorescent product, DCF, can undergo further oxidation to a phenoxyl-type radical ($\cdot\text{DCF}$) or photosensitization to DCF^* for oxidizing reductants, yielding additional $\cdot\text{O}_2^-$ and H_2O_2 that promote additional production of DCF from DCFH. The mechanism explains why the slopes in Fig. 4a gradually decrease over time. The larger slopes in the beginning are attributed to the relatively larger molar amount of oxygen initially dissolved in DCFH sample for greater production of DCF. The

progressive depletion of oxygen results in the slopes' asymptotic decrease. The nearly constant final small slopes are associated with constant oxygen supply from air significantly limited by diffusion through oil cap.

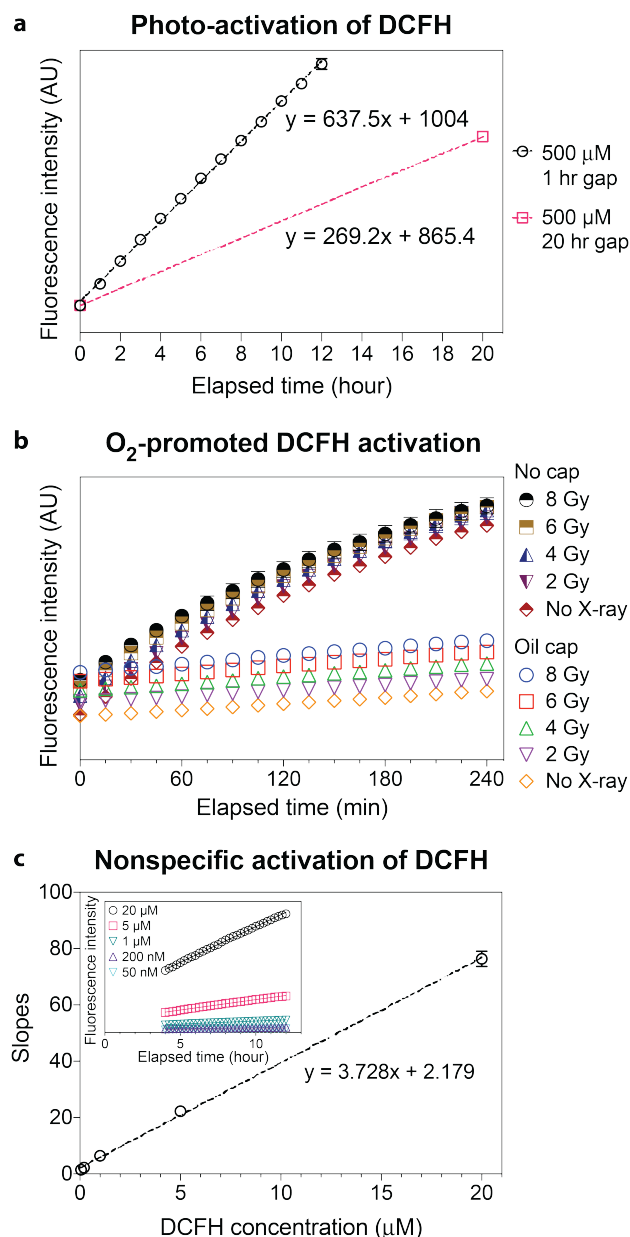


Figure S4. Investigation of nonspecific activation of DCFH via kinetic fluorescence spectroscopy. (a) Comparison of fluorescence increase rate using two different time intervals in spectroscopy. (b) Comparison of fluorescence change in time between two samples, one covered with oil and the other with no oil cap. (c) Slopes obtained via linear regression of the data shown in Fig. 4a as a function of DCFH concentration of the

samples. Only the data of 4 – 12 elapsed time which exhibit linear increase in fluorescence were used for slope calculations. As discussed in the main manuscript, we excluded the data measured in earlier times because we believe the nonlinear fluorescence change was influenced by the oxygen initially dissolved in DCFH samples.

G. Protocols of preparing 10 μM DCFH from DCFH-DA

The protocol Ha et al. employed to prepare DCFH from DCFH-DA for flow radiocytometry experiments is as follows:

- (1) Dissolve 0.025 g of DCFH-DA into 5 mL of methanol (10 mM DCFH-DA)
- (2) Add 15 μL of this 10 mM DCFH-DA solution to 60 μL of 0.01 N NaOH contained in a 15 mL Falcon tube.
- (3) Keep it at dark and room temperature for 30 min
- (4) Add 1025 μL of pH 7.4 10X PBS to the DCFH-DA/methanol/NaOH solution
- (5) Add 13.9 mL of pH 7.4 1X PBS to the DCFH-DA/methanol/NaOH solution (final DCFH-DA 10 μM , NaOH 40 μM)
- (6) Degas using sonication for 2 min followed by centrifugation to remove bubbles

H. Influence of BSA, EDTA, Mg^{2+} to radiofluorogenic conversion of DCFH

The dispersed phase comprises the mixture of equal volumes of 10 μM DCFH solution and cell suspension in Cell Dissociation Medium Type II. We aimed to assess influence of adding BSA, EDTA, and Mg^{2+} to the DCFH solution upon radiofluorogenesis and find adequate concentrations of these chemicals for Cell Dissociation Medium Type II. We prepared eight samples: 5 μM and 0.5 μM DCFH solutions and the DCFH solutions

added with either Mg^{2+} , EDTA, or BSA. We performed kinetic fluorescence spectroscopy, and the results are shown in Fig. S5. Notably, the addition of EDTA significantly reduced the fluorescence, which was also observed in another spectroscopy experiment for the mixture samples of DCFH, EDTA, and FDG. EDTA is known to chelate transition metal ions such as Cu^{2+} , Fe^{2+} , or Zn^{2+} and prevent Fenton reaction, which results in the creation of hydroxyl radicals from hydrogen peroxide through catalysis by transition metals that have a free coordination site. One-electron oxidizing species such as hydroxyl radicals are highly potent in converting DCFH to fluorescent DCFH. Therefore, we assume that EDTA suppressed the radiofluorogenic conversion of DCFH by preventing the production of hydroxyl radicals. Additionally, EDTA may have quenched some ROS such as $\cdot\text{DCFH}$, $\cdot\text{DCF}^-$, $\cdot\text{OH}$, and $\cdot\text{O}_2^-$ and interrupted radiofluorogenic conversion of DCFH. For these reasons, we excluded EDTA from Cell Dissociation Medium Type II as it significantly inhibited radiofluorogenesis.

In another spectroscopy experiments, the mixture samples of DCFH and FDG added with BSA exhibited higher SBR than those with no BSA. The results are shown in Fig. S6. We added 3 μM of BSA in Cell Dissociation Media Type II. We added magnesium ions to activate DNase I in the

media with the lesser amount (i.e., 2.5 mM) compared to the type I because there is no EDTA to chelate the magnesium ions.

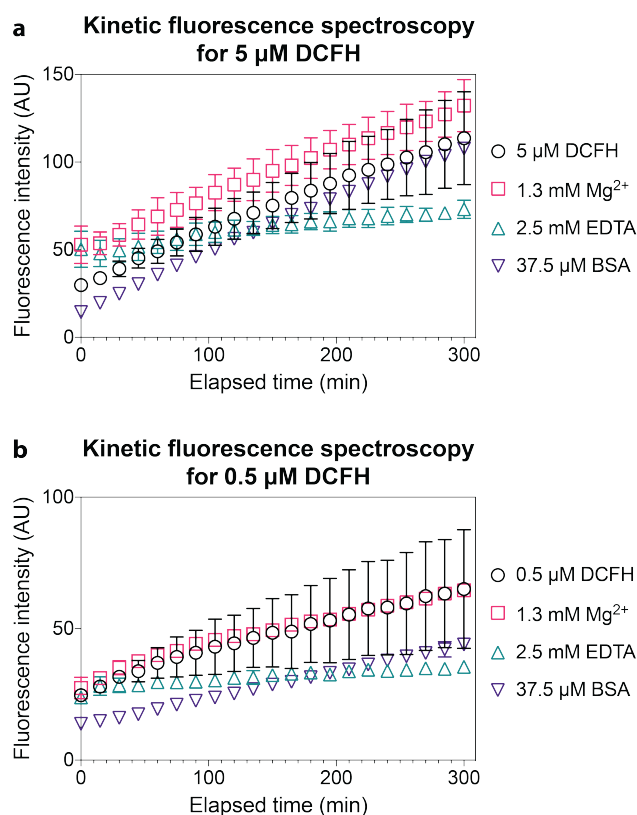


Figure S5. Kinetic fluorescence spectroscopy measurements of the following samples: (a) 5 μM DCFH, 5 μM DCFH added with 1.3 mM Mg^{2+} , 5 μM DCFH added with 2.5 mM EDTA, and 5 μM DCFH added with 37.5 μM BSA. (b) 0.5 μM DCFH, 0.5 μM DCFH added with 1.3 mM Mg^{2+} , 0.5 μM DCFH added with 2.5 mM EDTA, and 0.5 μM DCFH added with 37.5 μM BSA. The fluorescence measurements show averages and standard deviations of 4 samples of 100 μL dispensed on a 96-well plate covered with paraffin oil on top.

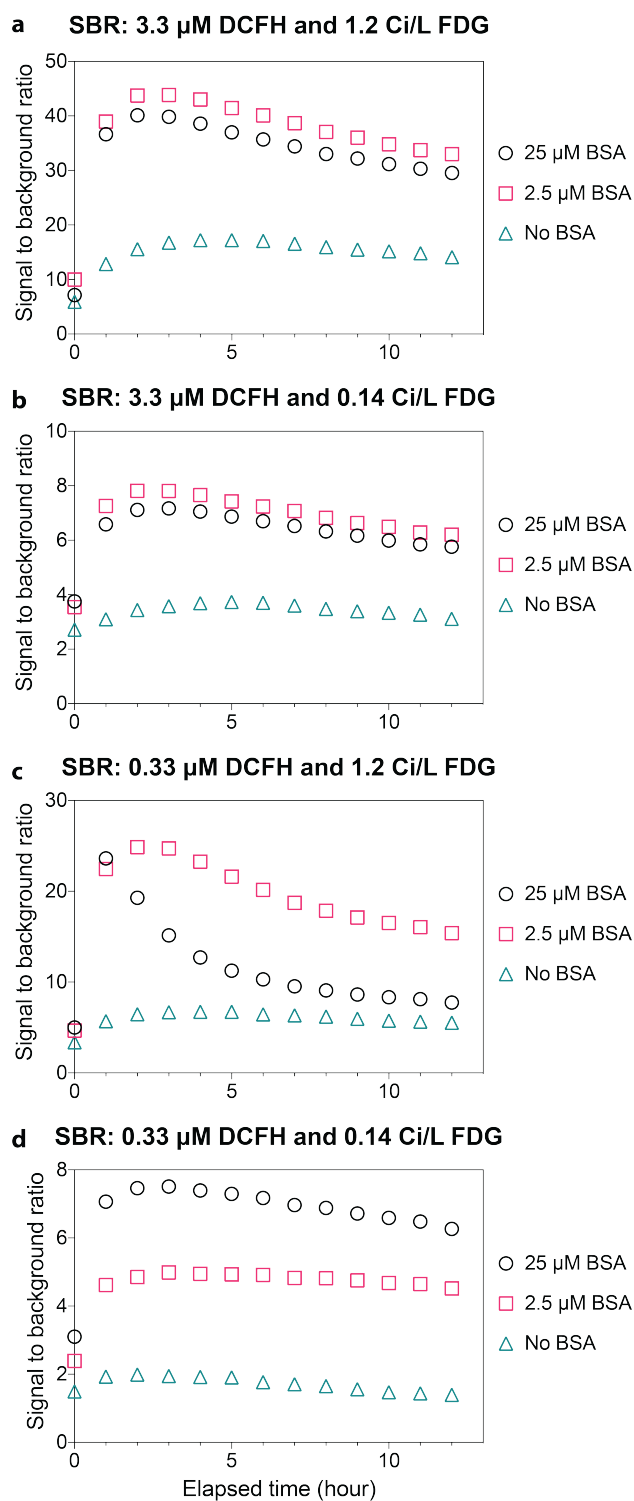


Figure S6. Signal-to-background ratio measured from kinetic fluorescence spectroscopy of DCFH added with FDG and increasing concentration of BSA: (a) 3.3 μ M DCFH and 1.2 Ci/L ($\alpha = 0.36$ Ci/ μ mole) (b) 3.3 μ M DCFH and 0.14 Ci/L ($\alpha = 42$ mCi/ μ mole) (c) 0.33 μ M DCFH and 1.2 Ci/L ($\alpha = 3.6$ Ci/ μ mole) (d) 0.33 μ M DCFH and 0.14 Ci/L ($\alpha = 0.42$ Ci/ μ mole)

I. Western Blot for GLUT1 expression

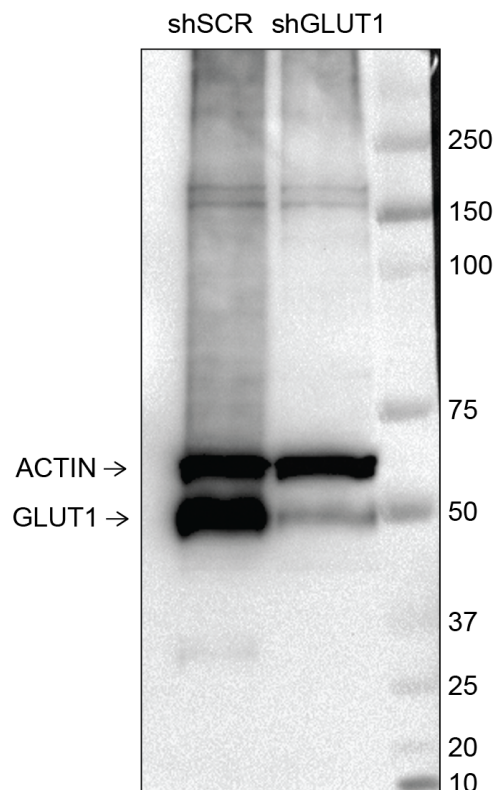


Figure S7. Uncropped western blot image of GLUT1 expression in MDA-MB-231 cells with GLUT1 knock-down or non-targeting control shRNA.