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Supplemental Information

Single-Cell Tracking of Breast Cancer Cells Enables Prediction of Sphere Formation from Early Cell Divisions

Patrick C. Bailey, Rachel M. Lee, Michele I. Vitolo, Stephen J.P. Pratt, Eleanor Ory, Kristi Chakrabarti, Cornell J. Lee, Keyata N. Thompson, and Stuart S. Martin

Figure S1:

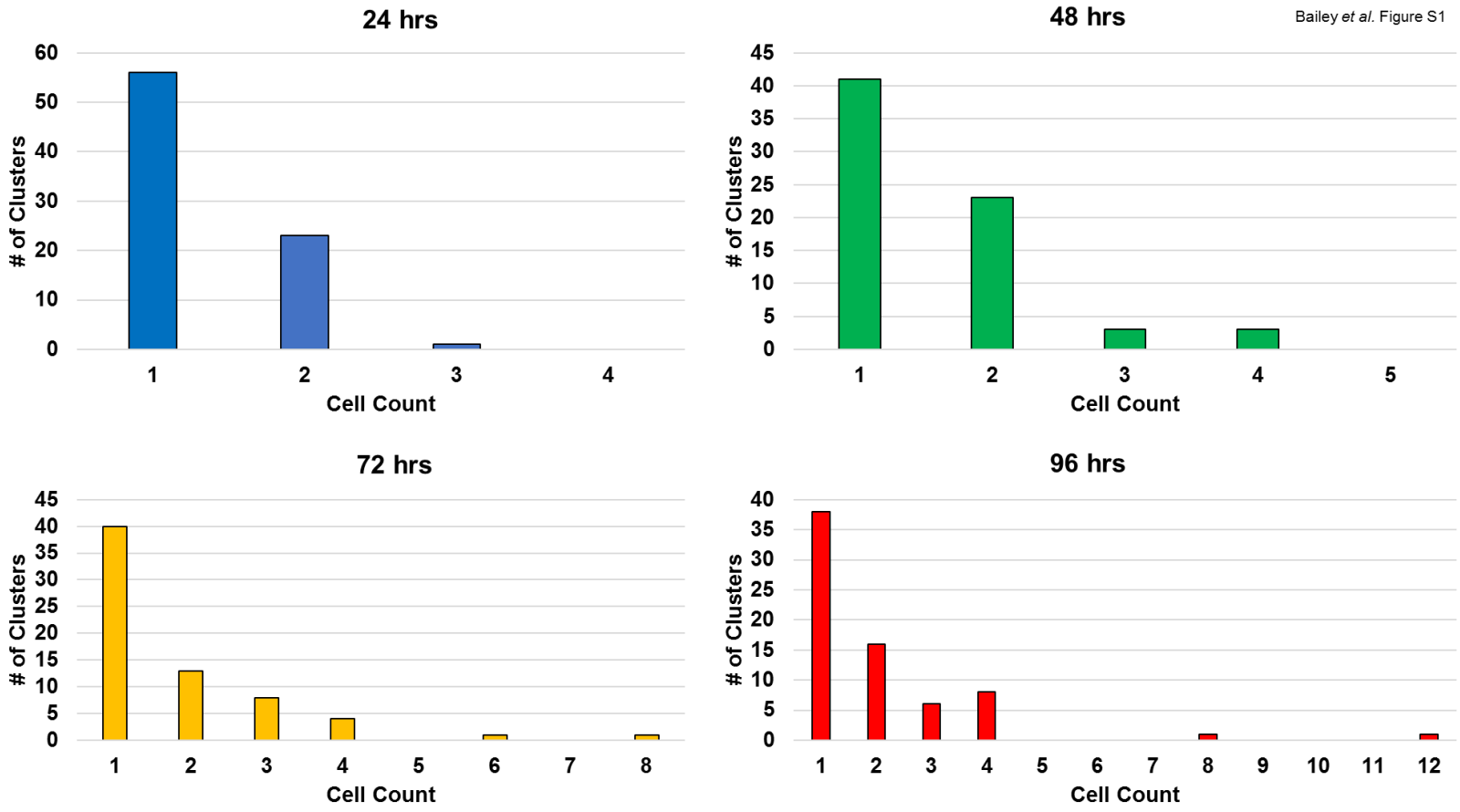

Figure S1: Long periods of time spent out of incubation ablates mammosphere growth Related to Figure 1. Progress of mammospheres grown from single cells FACS sorted into 96 well plates. PreSp cell counts on the X axis, number of PreSp for each cell count on Y axis. Graphs for 24, 48, 72 and 96 hours are shown. All PreSp arose from single cells (N=2).

Figure S2:

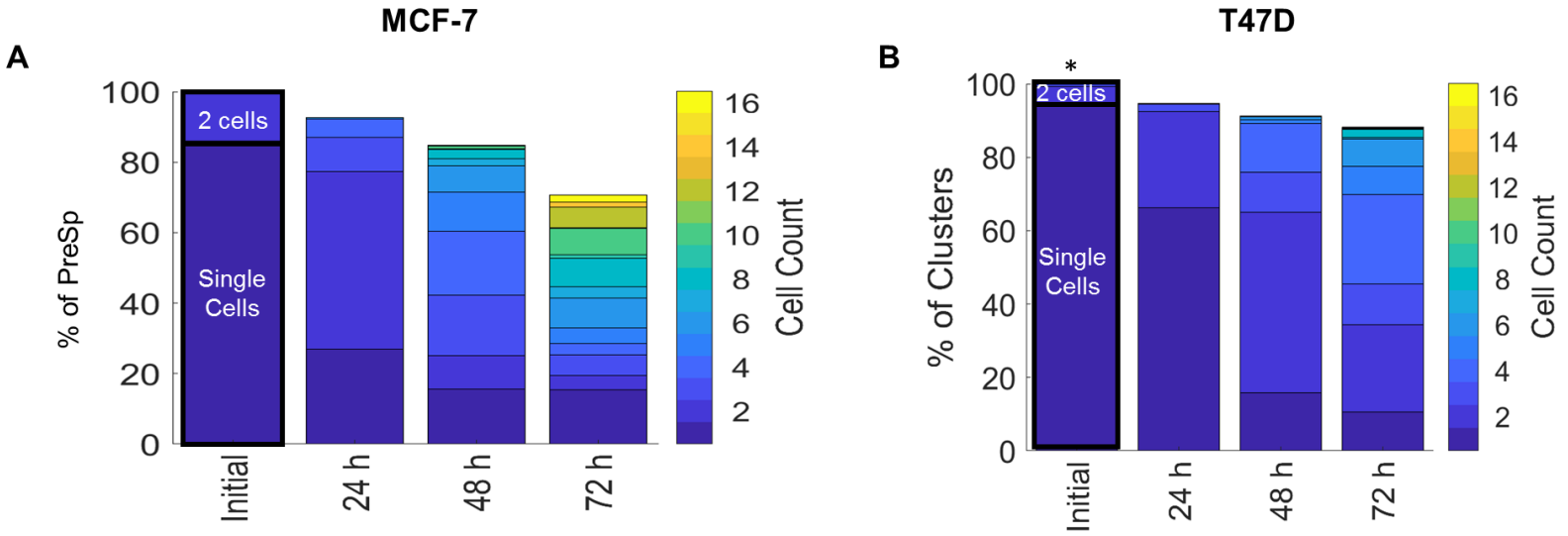

Figure S2: Size information of PreSp for first 72 hours of tracking. Related to Figure 1.
A,B) Cell count information for first 72 hours of mammosphere assay. Color indicates cell number in individual clonal outgrowths, bar height indicates percentage of total cllls. Percentages normalized to initial cell count (*=13, 2-cell clusters were manually removed from T47D wells and were thusly not tracked).

Figure S3:

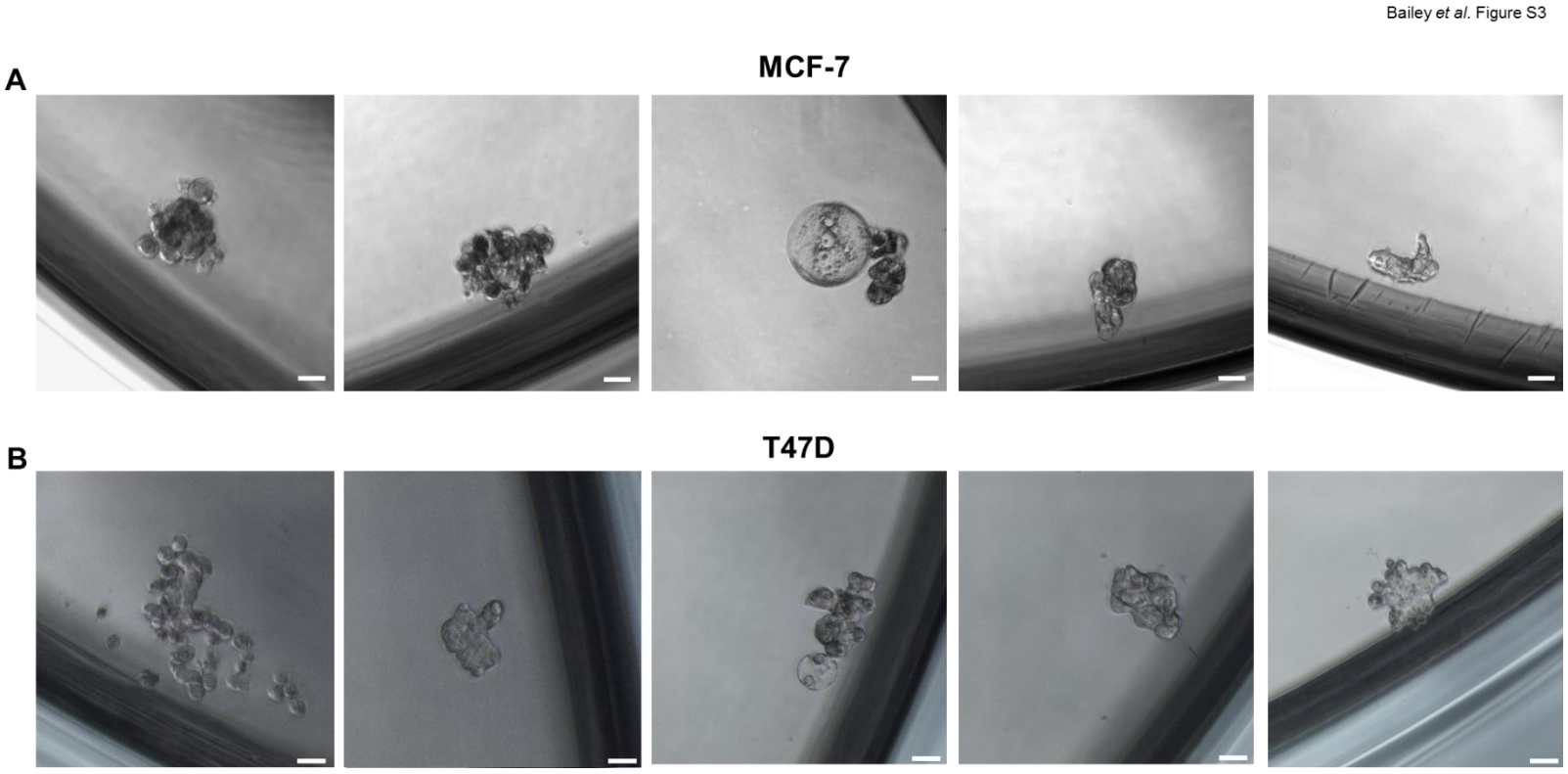

Figure S3: Clonal spheres are not identifiable by shape. Related to Figure 3. A) Various morphologies of clonal MCF-7 mammospheres at 7 days. **B)** Various morphologies of T47D mammospheres at 14 days. Scale bars are 20um. Related to Figure 3

Figure S4:

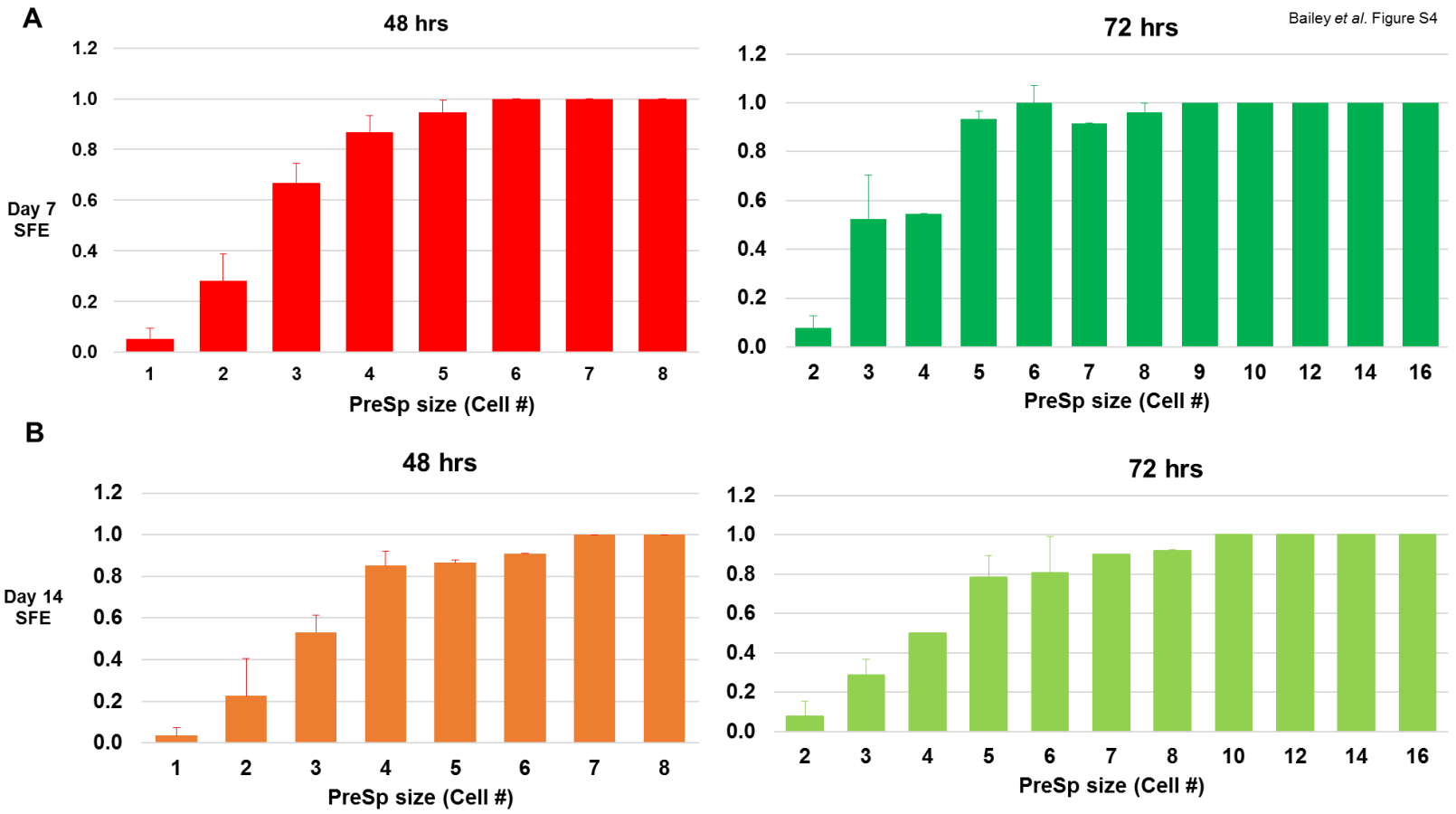

Figure S4: **Predictive capacity of early PreSp size. Related to Figure 4. A)** Single MCF-7 cells were tracked over the course of 14 days and early size information was correlated to eventual sphere formation. PreSp size at 48 and 72 hours was correlated to sphere formation on day 7 and expressed as a ratio of total clusters to spheres. Spheres were considered to be objects over 50μm. **B)** Same as A, save spheres over 50μm on day 14 were counted. Related to Figure 4

Figure S5:

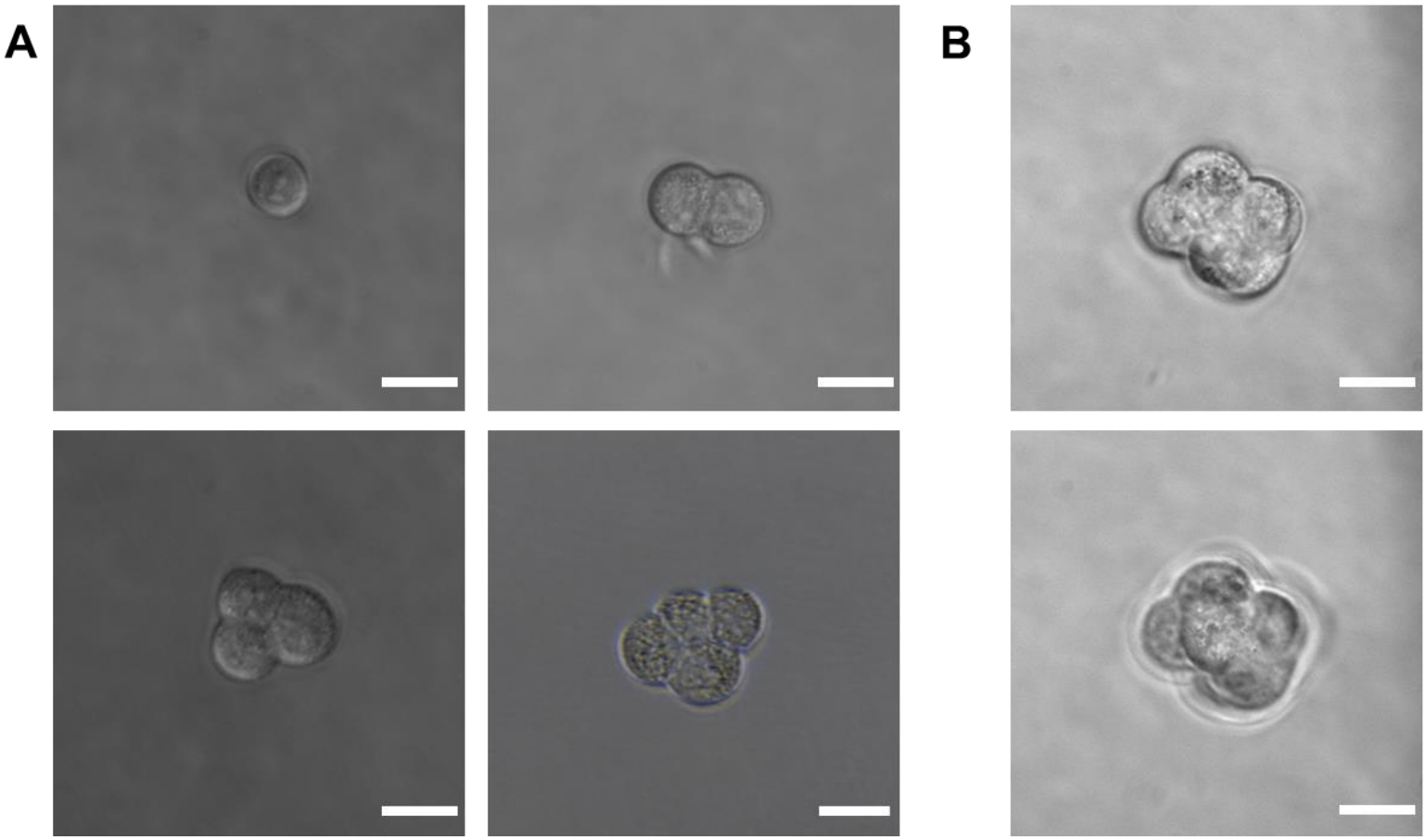

Figure S5: Cell counts at 24 hrs. are unambiguous while later days are more subjective. Related to Figure 4 A) Photographs at 40X of PreSp grown in suspension for 24 hours. Counts of 1, 2, 3 and 4 cells are clearly defined. Scale bar is 20µm. **B)** Photograph at 40X of PreSp grown in suspension for 72 hrs. A count of six cells was determined for this cluster by focusing up and down through the sphere (Upper and lower panels are two different focal planes).

TRANSPARENT METHODS

Cell culture: MCF-7 cells were cultured in DMEM media with 4.5g/L glucose and L-glutamine supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. Cells were maintained at 37ºC with 5% CO2 and passaged at 80% confluency.

Mammosphere culture and cell tracking: Cells were trypsinized, triturated repeatedly and passed through a 40um cell strainer to enrich for single cells. Cells were counted and visualized on a hemacytometer. If cell clumps were observed, cells were passed through a 25-gauge needle 10 times. For density dilution assays cells were seeded in 1 mL Mammocult media supplemented with heparin, penicillin/streptomycin and hydrocortisone per manufacturers instruction (complete media) in 24 well Ultra-low Attachment plates and incubated at 37ºC 5%CO2 for 7 days. Each density was seeded in triplicate for one experimental replicate (N) for a total of three replicates. For tracking and predictive experiments cells were diluted to 2 cells/300 µL in complete Mammocult media. Cells were seeded in the center 12 wells of 96 well Ultra-Low Attachment plates at a volume of 300µL per well. The outside edges of the plates were filled with PBS to prevent evaporation of media. Each experiment used 8 plates with 12 wells seeded. Cells were allowed to settle in an incubator for 2 hours, after which light microscopy was employed to visualize the position of every cell in the plate (No specialized equipment required). These initial positions were marked for subsequent tracking. Manual manipulation with a 10µL pipette was employed to move cells that were either too numerous or too close together. Every day for 7 days and on days 10 and 14 PreSp position, count, size and morphology were recorded. Both tracking and predictive experiments were repeated three times.

Flow Cytometry and FACS: Cells were trypsinized, triturated repeatedly and passed through a 40um cell strainer to enrich for single cells. Cells were counted using a hemacytometer and centrifuged for 5 minutes at 300g. Cells were washed one time with Flow Buffer (1% BSA, 10mM EDTA in PBS) and spun down again. Cells were resuspended at a density of 10^7 cells/mL in Flow Buffer and 100µL aliquots were made. APC

conjugated CD44 and FITC conjugated antibodies (Biolegend) were added simultaneously (after confirming no difference compared to one at a time) according to manufacturers recommendations. Aliquots were incubated for 20 minutes at 4^{o}C in the dark. Aliquots were spun down and washed 2X with Flow Buffer. Cells were resuspended in 1mL Flow Buffer and passed through a 40µm cell strainer for subsequent flow cytometry. Sorting and analysis were performed on a FACS Aria II and cells were sorted to ultimately achieve 10 cells/well. After sorting every individual cell was counted to insure accurate SFE calculations.

Data analysis: A custom MATLAB program and Excel spreadsheet were employed to tally and compile tracking data and prediction information. Data compilation was performed in each program independently and used to validate the results of the other. SFE is calculated as (total spheres/total cells tracked)*100 . Unless otherwise noted, bar graphs represent the weighted average of the experiments with the number of PreSp or number of spheres used for the weight as appropriate. Error bars show weighted standard deviations unless otherwise noted.