SUPPORTING INFORMATION

NANOCHIPS ASSISTED PEPTIDE SCREENING FOR CLINICAL DEVELOPMENT OF CAR-T CELL IMMUNOTHERAPY

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1) In-house synthesis of M0 and M-peptide sequences

E5B9 peptide variants **A0**-**A2** were purchased from Biosyntan LtD. Peptides **M0**, **M1-M3** were synthesized in an automated microwave peptide synthesizer (Biotage Initiator+ Alstra) using the Fmoc/*t*Bu strategy. 141 mg of Fmoc-Rink-Amid MBHA resin (0.71 mmol/g, 0.1 mmol) were swollen in DMF at 70°C for 30 min. For **M1** and **M2**, Fmoc deprotection was done by treatment with 20% piperidine/DMF (2×10 min) and for **M0** and **M3** (due to the presence of Asp) with 0.1 M HOBt in 20% piperidine/DMF. The resin was washed two times with DMF. Coupling of the next amino acids was performed by addition of DIPEA (10 eq.), amino acid (5 eq., 0.2 M in DMF) and HATU (5 eq., 0.5 M in DMF) and microwave irradiation for 5 min at 80°C. In case of Fmoc-Cys(Trt)-OH, coupling was conducted at room temperature for 60 min according to the manufacturer instructions. After each coupling cycle, the resin was washed two times with DMF. After complete assembly of the linear peptides, the resin was washed manually with DMF, CH_2Cl_2, CH_3OH and Et_2O two times each. For cleavage from the resin and concomitant removal of the side-chain protecting groups, 0.5 mL H_2O, 0.5 mL TRIS and 10 mL TFA was added (in that order) to the dry resin and the mixture was stirred for 2 h. The resin was rinsed once with 5 mL TFA and the combined eluates (in a 50 mL Falcon tube) were concentrated under vacuum and nitrogen flow. The residue was taken up in ice-cold Et_2O and the mixture was stored at -20°C over night. The suspension was centrifuged at 7,830 rpm for 5 min, the supernatant was decanted and 10 mL of ice-cold Et_2O was added to the pellet. This procedure was repeated three times. Finally, the pellet was dried in an oven at 55°C for 30 min. The crude products were purified by preparative RP-HPLC. The peptides **M0** and **M1-M3** were obtained as white fluffy solids in excellent chemical purity (≥98% @230 nm).

Analytical and preparative RP-HPLC was performed with a Shimadzu system L-20A Prominence equipped with a UV detector (SPD-M20A), two pumps (LC-A20R), a degasser (DGU-20A5R), a column oven (CTO-20AC), a communication module (CBM-20A) and a fraction collector (FRC-10A). A complementary pair of columns (Phenomenex Jupiter Proteo, 4 µm, 90 Å with dimensions of 250×4.6 mm and 250×21.2 mm) was used. A binary gradient system of 0.1% TFA/water (solvent) and 0.1% TFA/CH_3CN (solvent B) at a flow rate of

1 mL/min or 10 mL/min served as the eluent. The identity of the peptides was proven by measuring low resolution mass spectra using electrospray ionization on a UPLC-DAD-MS system from Waters (ACQUITY UPLC I-Class System including a ACQUITY UPLC eλ-Detector coupled to a Xevo TQ-S mass spectrometer). A binary gradient system of water (containing 0.1% CH_3COOH) as solvent and CH_3CN/CH_3OH (1:1, containing 0.1% CH_3COOH) as solvent B at a flow rate of 0.4 mL/min served as the eluent. As stationary phase a Acquity UPLC BEH C18 column (waters, 100 x 2.1 mm, 1.7 µm, 130 Å) was used.

A phosphate-buffered saline (PBS) solution (pH 7.4) was prepared from dry tablets (Sigma Aldrich) and laboratory de-ionized water.

Code	**Amino acid sequence**	
A0	**SKPLPEVTDEY** -OH	BIOSYNTAN Ltd, Germany
A1	**SKPLPEVTDEY**-PEG_2-PEG_2-NH-$(CH_2)_2$-SH	BIOSYNTAN Ltd, Germany
A2	3-mercapto propionic acid -PEG_2-PEG_2- **SKPLPEVTDEY**-OH	BIOSYNTAN Ltd, Germany

Code	**Amino acid sequence**	**m/z calculated for $[M+H]^+$**	**m/z found for $[M+H]^+$ [a]**	**Purity (%)[b]**
M0	C**SKPLPEVTDEY**-$CONH_2$	1379.65	1380.0	≥99
M1	C**SKPLPEVTGEY**-$CONH_2$	1321.65	1322.1	≥98
M2	C**SKPLPEVTGGY**-$CONH_2$	1249.62	1250.1	≥98
M3	C**SKPLPGVTDGY**-$CONH_2$	1235.61	1236.1	≥98

a) Low resolution mass spectra using electrospray ionization were recorded. b) Purity was determined by analytical RP-HPLC and is given for 230 nm

2) Contact angle measurement

For this experiment, we use 1cmx1cm piece of silicon wafer and apply the same functionalization protocol as described in the main text. The surface of the samples are characterized by contact angle measurement immediately after each step. Static contact angle measurements are recorded by dropping 5 uL of deionized water on the sample's surface and

analyzed with sessile drop method (Young-Laplace fitting). The measurements are performed in room temperature by drop shape analyzer DSA25 (KRUSS GmbH, Germany) and the values shown in the paper are the averages of five measured values.

3) Fluorescent microscopy test

To visually confirm the functionalization process, we take fluorescence images during each step of the procedure. A secondary Ab labeled with Alexa 488 (Goat anti-Mouse IgG A-11001, Thermo Fisher Scientific) is used to verify the presence of the anti-E5B9 mAb. The dye has maximum excitation and emission wavelength of 490 nm and 525 nm, respectively, which can be detected by most conventional GFP filter set. For better image contrast, we create a 50 µm-hole pattern with maN1420 photoresist using UV-lithography. Firstly, microscope glass coverslips are cleaned according to RCA-1 and RCA-2 protocol to remove any organic and ionic residues. A layer of promoter AR300-80 is spun before maN1420 to improve the photoresist's adhesion to the glass surface. The hole-pattern is then transferred from the photomask to the glass slide by exposure to UV light. The functionalization protocol as described in the Material and Methods is applied to functionalize the glass slides with a photoresist pattern with an additional step to remove the resist right after TESPSA self-assembly. The coverslips are placed in acetone in an ultrasonic bath for 15 minutes to lift-off the resist. After that, the glass slides are rinsed with IPA, blown dry, and cured on a hotplate at 120°C for 30 minutes. For this testing, WT E5B9 peptide was immobilized on the glass surface. After blocking with ethanolamine, we incubate the glass pieces with anti-E5B9 mAb at a high concentration of 0.1 µg/mL for 30 minutes to ensure all active sites are saturated. Next, the glass slides are washed several times with 1xPBS to remove any unbound mAb. In a dark box, we then incubate the glass slides with fluorescent secondary antibodies (0.1 µg/mL) for 30 minutes and again carefully wash the slides with 1xPBS. After drying compressed air gun, images are taken by ZEISS microscope with 1200 ms exposure time and processed for false color expression by open-source software ImageJ.

4) Calibration curve of ELISA for 7 peptide derivatives

In ELISA assay, mAb was titrated from 0.1 ng/mL to 5 µg/mL. In nanosensor, mAb was titrated from 0.1 fg.mL to 1 ng/mL. Full evaluation and comparison of the two method performance are shown in the maintext (Fig.3, Table 1)

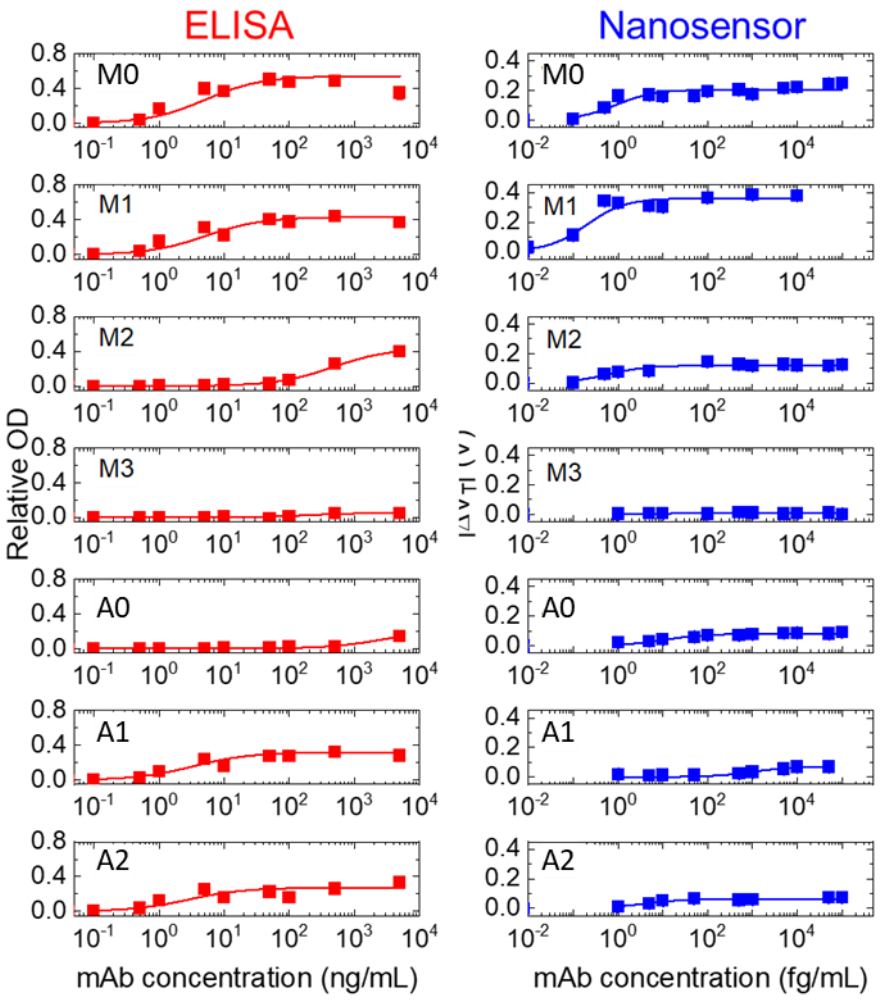

Figure S4: Calibration curves of ELISA and SiNW sensors for 7 peptides derivatives

5) Pearson Correlation test

The SiNW FET technology resulted in apparent K_d values with four to seven magnitude lower than those of ELISA. The phenomena is also observed in another study [1] where the resulted apparent K_d from the SiNW sensor was approximately six orders of magnitude lower than from surface plasmon resonance method. As K_d strongly depends on the limit of detection and saturation point of binding curve, the lower apparent K_d is contributed not only because of extremely sensitive detection capability of SiNW FET, but is also a consequence of limited sensing area leading to quick saturation and thus potential misinterpretation in the K_d definition. We therefore do not use the dissociation constant as a parameter in SiNW FET analysis due to the lack of reliability for binding strength evaluation.

Here we extracted four sensing performance parameters as illustrated in below figure for all the calibration curves. Smax presents maximum signal or average value from the saturation region of the curve. Kd shows concentration that give signal equal to half of Smax. Sensitivity extracted by getting the slope of linear region of the curve. Finally, limit of detecion is determined as the concentration that can produce a readout signal three times larger than the error of blank sample (withou anti-E5B9 mAb).

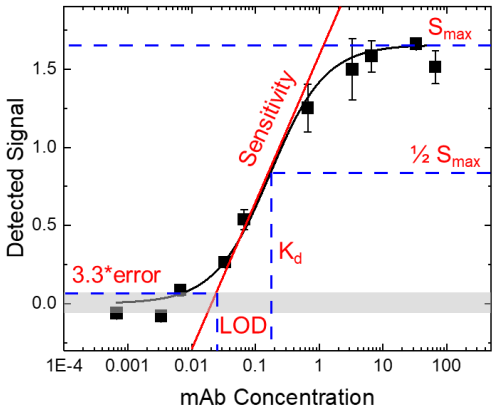

Figure S5.1: Extraction of sensing performance parameters

to compare and find a representative parameter for both ELISA and SiNW sensor method, we perform Pearson correlation test for the 2 sets of data (ELISA and SiNW sensor). The correlation coefficient is calculated by the equation:

$$r = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum(x_i - \bar{x})^2 (y_i - \bar{y})^2}}$$

where x are values of performance parameters of all peptides/M0-M group (S_{max}, Sensitivity, LOD or K_d) resulted from ELISA and y are values from SiNW sensors. Note that this Pearson correlation test is different from one presents in Figure 4.C in the main text where it compares complete calibration curves in the same methods but different sample media. Among four parameters of SiNW FET, the sensitivity give highest correlation, especially within M group of peptides. Moreover, the sensitivity of both methods are highly correlated in matrices thus can used as a common evaluation parameters in this study.

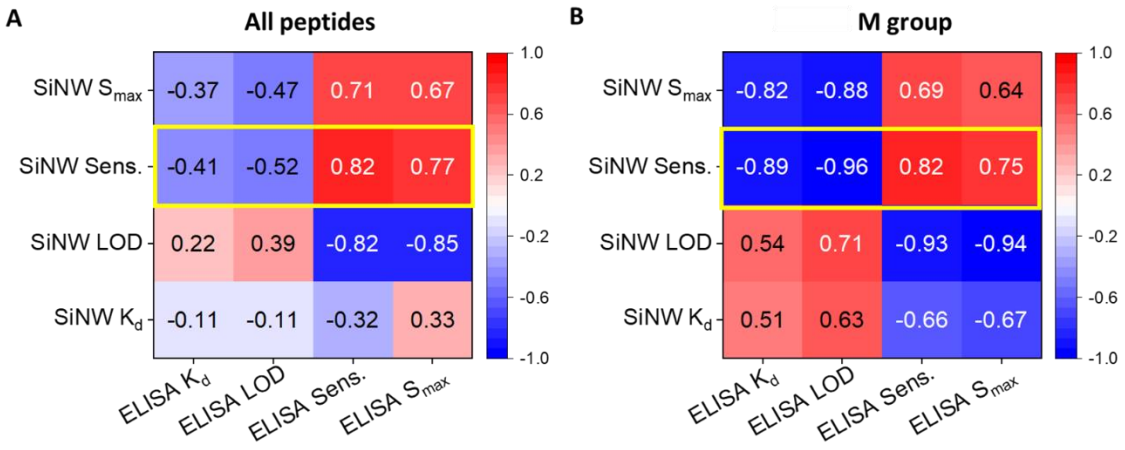

Figure S5.2: Pearson correlation matrices of performance parametes extracted from SiNW sensor and ELISA for (A) all peptides, (B) M group

6) Response of SiNW FET to serum sample

After sufficient washing step, serum at any tested concentration produces an unchanged V_G shift around 40mV. Addition of anti-E5B9 mAb increases the shift significantly. However, the amount of shifting depends on serum concentration.

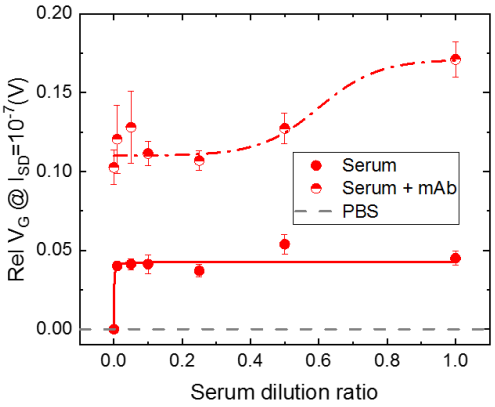

Figure S6: Calibration of serum dilution (filled circle) on a SiNW FET with M0 peptide functionalization. A fixed mAb concentration of 0.5 ng/mL is then added to each dilution of serum sample (half-filled-circle).

7) Comparison of BSA solution and serum

To better understand the serum contribution to the signal, we do the reference testing of the SiNW FET with the pure bovine serum albumin (BSA) (Figure S7). This shows that the most of signal contribution in serum might be associated to albumin binding, which make up more than half of protein components in human blood serum. A normal albumin range in adult human serum is 3.5-5.4 g/dL [2]. Therefore, we prepare a 3%w BSA in PBS solution to replicate

serum composition. At low concentrations, SiNW FET calibration shows that BSA solution responses similar to diluted serum. However, as concentration grows, the variation increases. Moreover, anti-E5B9 mAb diluted in BSA does not create as high V_G shifting as in PBS or in serum.

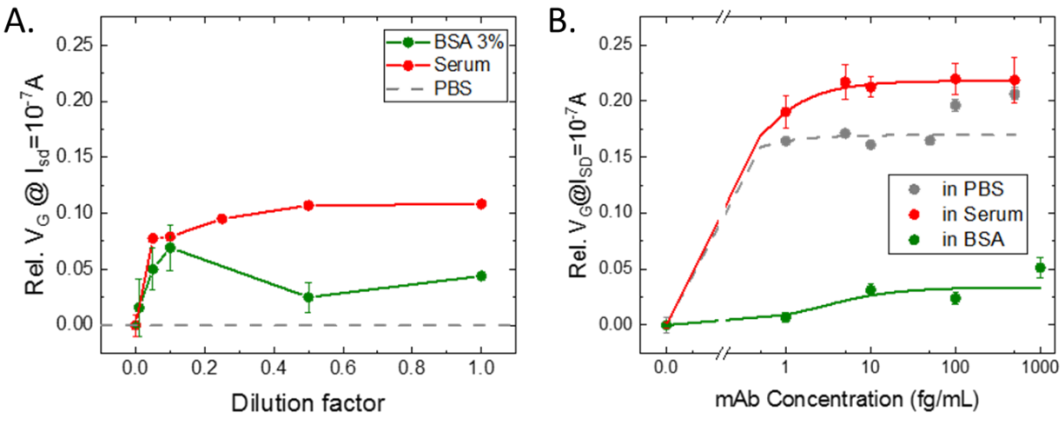

Figure S7: (A) Calibration of serum (red) and BSA (green) 3% solution at different concentration; (B) Calibration of mAb in PBS (grey), 0.1x Serum (red) and BSA 0.3% (green) in M0 functionalized sensor.

8) TWEEN test

To this extent, the serum protein can be easily removed by adding surfactants, such as for example TWEEN 20, which approximates the serum signal to its previous PBS level (FigS8 A). However, binding calibration curve of M0 and M2 peptides showed that addition of TWEEN 20 might reduce the obtained signal. Therefore, we decided to worked with only diluted serum.

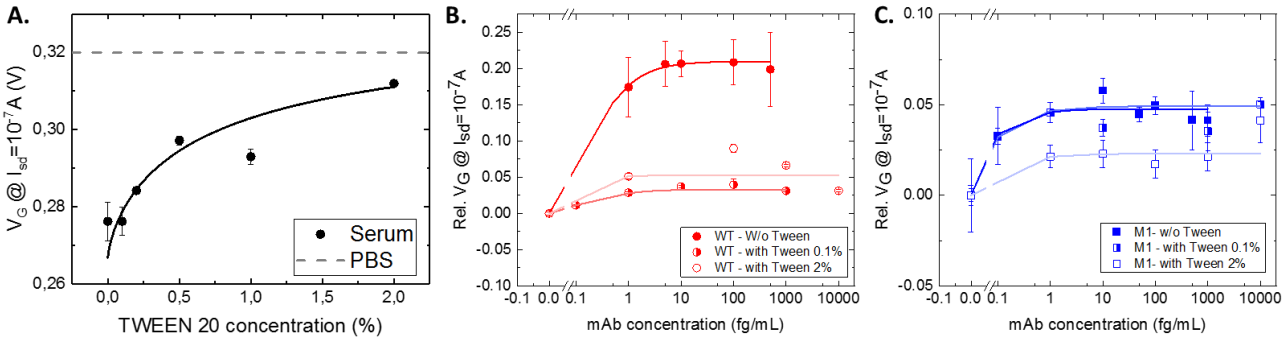

Figure S8: (A) Calibration of 0.1x serum with different TWEEN 20 concentration; Calibration of mAb in 0.1x serum (filled symbol), 0.1x serum with 0.1x TWEEN (hal-filled symbol), and 0.1x serum with 2% TWEEN 20 (empty symbol) for SiNW FET functionalized with M0 peptide (B) and M2 peptide (C).

9) Cytometry analysis of TM binding to PC3 cells, anti-La E5B9 and UniCar-T cells.

The interaction between the TM's C-terminal E5B9 peptide epitope and its respective anti-E5B9 mAb was also analyzed by flow cytometry. As shown in Figure S10, in general all PSCA-TMs could be detected on PSCA-expressing PC3-PSCA tumor cells via their C-terminal His-Tag proving that all different PSCA-TMs were functional. More interstingly, all E5B9 peptide epitope variants could be recognized by the corresponding anti- E5B9 mAb while the TM was binding on PC3-PSCA tumor cells. However, the recognition of the E5B9 M2 and M3 by the anti- E5B9 mAb was reduced in comparison to the E5B9 WT and M1. Differences in binding capabilities were even more pronounced when detecting the binding of the TMs on UniCAR-T cells (bottom line Figure S10). Only the binding of PSCA-TM-WT and PSCA-TM-M1 could be detected on UniCAR-T cells via their His-tag. In contrast, binding of the PSCA-TM-M2 and PSCA-TM-M3 were not detectable on UniCAR-T cells. The most likely reason for this is that binding affinity of the UniCAR anti-E5B9 scFv towards the mutated E5B9 M2 or M3 was reduced because of the mutations within the E5B9 sequences.

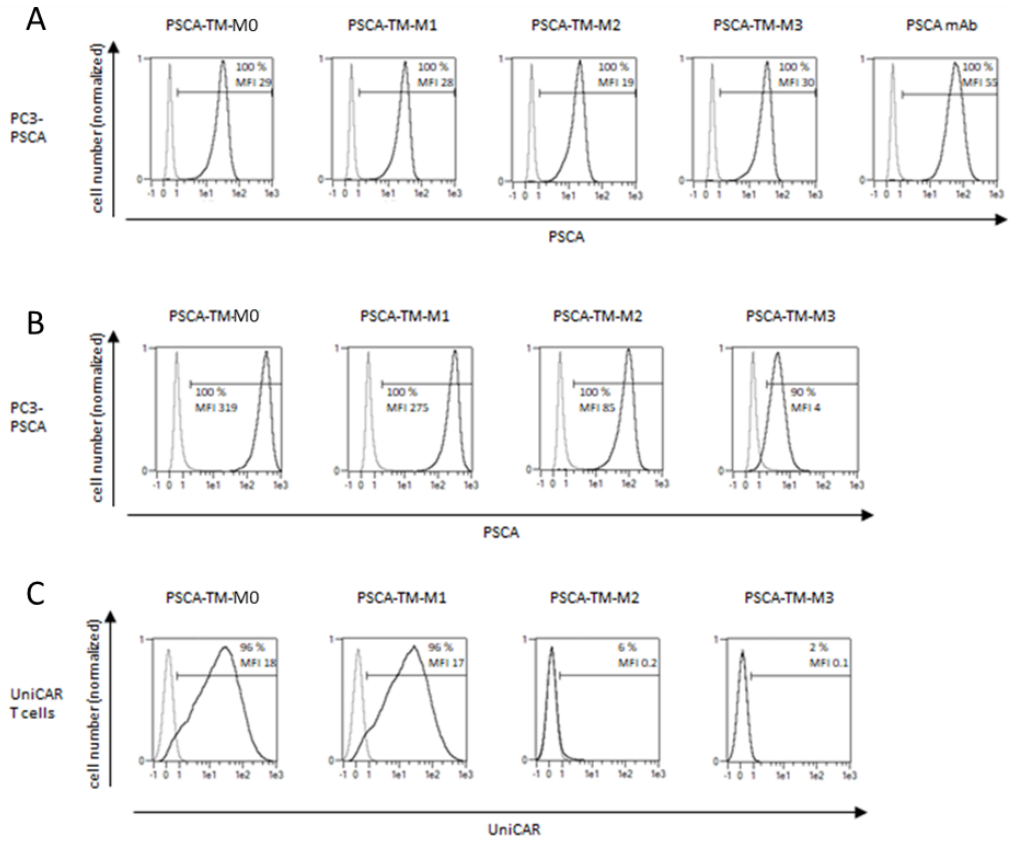

Figure S9: (A, B) Tumor cells or (C) UniCAR T cells were incubated with 1 µg (20 ng/µl) of TM. Binding was either detected via (A, C) the His-tag, using an anti-His-PE Ab or (B) the E5B9 epitope using the anti-E5B9 mAb and an anti-mouse IgG-PE Ab. (A-C) Stained cells are

shown in black, the negative control is shown in grey. Numbers in the histograms represent the percentage of positively stained cells, as well as the median fluorescence intensity (MFI).

References:

1. Zhang, H.; Kikuchi, N.; Ohshima, N.; Kajisa, T.; Sakata, T.; Izumi, T.; Sone, H. *ACS Applied Materials & Interfaces* **2020,** 12, (46), 51808-51819.
2. Davy, C. W.; Jackson, M. R.; Walker, J. *Lab Anim* **1984,** 18, (2), 135-42.