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Supplementary online material for:

Affibody-Targeted Fluorogen Activating Protein for *in vivo* Tumor Imaging

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Material and Methods

Preparation of probes. AffiFAP targeting probes were constructed as one dL5** flanked by two copies of affibody with an additional C-terminal cysteine. EGFR affibody ($Z_{EGFR:1907}$) and HER2 affibody ($Z_{HER2:342}$) [1-3] were used in this experiment. Cy5-maleimide was conjugated to the EGFR-specific affiFAP through the C-terminal cysteine. Cloning, purification and conjugation were described previously[4]. Sequences below show affibody amino acids underlined and FAP amino acids in boldface.

Her2 affiFAP: Addgene 85624 ($Z_{Her2-342}$, **FAP dL5****)

GPSKLASVENKFNKEMRNAYWEIALLPNLNNQQKRAFIRSLYDDPSQSANLLAEAKKLNDAQAPK**QAVVTQEPSVTVSPGGTVILTCG SGTGAVTSGHYANWFQQKPGQAPRALIFDTDKKYSWTPGRFSGSLLGAKAALTISDAQPEDEAEYYCSLSDVDGYLFGGGTQLTVL SGGGGSGGGGSGGGGSGGGGSQAVVTQEPSVTVSPGGTVILTCGSGTGAVTSGHYANWFQQKPGQAPRALIFDTDKKYSWTPG RFSGSLLGAKAALTISDAQPEDEAEYYCSLSDVDGYLFGGGTQLTVLS**GSTSGTVENKFNKEMRNAYWEIALLPNLNNQQKRAFIRSLY DDPSQSANLLAEAKKLNDAQAPK

EGFR affiFAP: Addgene 73217 ($Z_{EGFR-1907}$, **FAP dL5****)

GPSKLASAEAKYAKEMWAAWEEIRNLPNLTGWQMTAFIAKLVDDPSQSSELLSEAKKLNDSQAPK**QAVVTQEPSVTVSPGGTVILTC GSGTGAVTSGHYANWFQQKPGQAPRALIFDTDKKYSWTPGRFSGSLLGAKAALTISDAQPEDEAEYYCSLSDVDGYLFGGGTQLTV LSGGGGSGGGGSGGGGSGGGGSQAVVTQEPSVTVSPGGTVILTCGSGTGAVTSGHYANWFQQKPGQAPRALIFDTDKKYSWTPG RFSGSLLGAKAALTISDAQPEDEAEYYCSLSDVDGYLFGGGTQLTVLS** GSTSGT AEAKYAKEMWAAWEEIRNLPNLTGWQMTAFIA KLVDDPSQSSELLSEAKKLNDSQAPK

Dye synthesis. MG-Btau was synthesized as described previously, without modification. [5]

Cell culture and tumor model. Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of Carnegie Mellon University. A431 cells (ATCC CRL-1555) were cultured in DMEM (Thermo Fisher) supplemented with 10% FBS (FisherBrand) at 37°C in humidified air containing 5% CO_2. EGFR-enriched tumor models were established in athymic nude mice (athymic nudes; Envigo). Suspended A431 cells ($5x10^6$ cells in 50-100 µL of PBS) were inoculated subcutaneously into the right thighs of the animals. Animals were used for experiment 10-15 days post-inoculation when tumors were >1 cm in size (length + width).

***In vivo* tumor imaging.** Probes were introduced to mice through tail-vein injection at a concentration selected to achieve a final dose of 200 nM in circulation. An IVIS Spectrum CT Optical Imaging System (Perkin Elmer) was used for *in vivo* imaging with an excitation filter of 640/20 nm and emission filter of 680/20 nm to capture tumor fluorescence images. Images obtained with 535/20 nm excitation and 680/20 nm emission filters were used as fluorescence background and subtracted. Once mice were sacrificed, organs were harvested for fluorescence detection. Tumors were weighed and cryogenically stored in OCT compound at -80°C.

Tumor section fluorescence imaging. Collected tumors were cyro-sectioned into 10 µm slices. The sections were stained with Hoechst and Alexa488-phalloidin and then mounted with PBS. The mounted tumor sections were imaged by a Nikon Eclipse Ti Slidescanning system with a 10x 0.5NA objective. Widefield fluorescence images were collected using appropriate spectral windows for each probe: 405 nm ex/460 nm em for Hoechst 33342, 480 nm ex/530 nm em for Alexa 488, 640 nm ex/680 nm em for MG and Cy5, and images were collected with a Hamamatsu Orca Flash 4.0 camera.

Probe half-life measurement. Swiss Webster female mice were used to determine the half-life of affiFAP and MG. A dose sufficient to achieve 200 nM of the final construct in the blood pool (assuming 1.5 mL total blood volume) was tail-vein injected and blood samples (typically 20ml) were collected by submandibular bleeding [6] using heparinized capillary tubes at different time points. Plasma was isolated using a plasma separation wax (from BD Vacutainer serum collection vials) and a hemocentrifuge. 5 µL of plasma was complexed with 15 µL of 50 µM dL5** or MG-Btau and the fluorescence was measured by Tecan Infinite M1000 plate spectrometer using 636nm excitation and 664nm emission.

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