ADVANCED BIOSYSTEMS

Supporting Information

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Modular Antigen-Specific T-cell Biofactories for Calibrated In Vivo Synthesis of Engineered Proteins

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Experimental designs and statistical analysis. The experimental design for **Figures S1-S3** is described. GraphPad Prism 6 (GraphPad Software, Inc) was used to conduct all statistical analysis.

i) ***Figure S1*** **(Design and selection of the *Actuator*).** The Jurkat Clone E6-1 suspension cell line was engineered with the lentivirus particles carrying three variations of the *T-cell Biofactory* insert (per **Fig. 1B** but without the *Secretor* domain and without the *Sensor-Receptor* domain). Each variation consisted of the *Effector* transgene regulated with a different version of the *Actuator* (three, six, and nine copies of NFAT-RE as per **Fig. S1(i)**). The Nluc activity for three *T-cell Biofactories* chemically stimulated (1 µM ionomycin, 30 nM phorbol 12-myristate 13-acetate (PMA) for 24 hrs) for $[Ca^{2+}]_i$ rise was fit using Y = a + b * log(X) where X is the number of *T-cell Biofactories* (**Fig. S1(ii)**) and Y = a + b * X + c * X^2, where X is the stimulation time in hrs (**Fig. S1(iii)**). The signal-to-noise ratio (S/N) for each *T-cell Biofactory* was determined by calculating the ratio of means of Nluc activity from the chemically activated *T-cell Biofactory* to that of its non-activated counterpart (**Fig. S1(ii)**). The error bars are one standard deviation (SD) above and below the mean and can also be considered as one half-width of an 86% confidence interval for that mean. The upregulation in Nluc activity was observed for at least 10 days. (**Fig. S1(iii)**). Although higher Nluc activity was observed from the *T-cell Biofactories* engineered with NFAT-RE9X, the NFAT-RE6X

Actuator was preferred due to its reduced potential to non-specifically activate and was selected for use in generating the *T-cell Biofactory* as shown in **Fig. 1A**.

ii) ***Figure S2*** **(Design and selection of the *Sensor-Receptor*).** The Jurkat Clone E6-1 suspension cell line pre-engineered with $_{\text{NFAT-RE6X}}\rightarrow$Nluc-2A-GFP was further engineered with lentivirus particles for three different variations of *Sensor-Receptor* under a neomycin resistance marker translated by aminoglycoside 3'-phosphotransferase transgene. Each variation consisted of a different version of the constitutively expressed *Sensor-Receptor* (as per **Fig. S2(i)**) for targeting FRa on the antigen-presenting cell. The three *T-cell Biofactories* (per **Fig. 1B** but without the *Secretor* domain) and a control (per **Fig. 1B** but without the *Secretor* domain and without the *Sensor-Receptor* domain) were biologically stimulated (using the three A2780cis variant cell lines engineered for FRa or MSLN expression for 24 hrs). The S/N for each *T-cell Biofactory* was determined by calculating the ratio of means of Nluc activity from the *T-cell Biofactory* stimulated by the antigen-positive cells ($FRa^{+}MSLN^{neg}$ A2780cis or $FRa^{neg}MSLN^{+}$ A2780cis) to that stimulated by the $FRa^{neg}MSLN^{neg}$ A2780cis cells (**Fig. S2(ii)**). The error bars extend 1 SD above the mean and can also be considered as one half-width of an 86% confidence interval for that mean. Based on these data, the FRa-CAR3 *Sensor-Receptor* was selected for use in generating the *T-cell Biofactory* as shown in **Fig. 1A**.

Compared to FRa-CAR2, the intracellular portion of the FRa-CAR3 contains the additional 4-1BB co-stimulatory domain found to enhance the therapeutic response of CAR T cells by mitigating early exhaustion of T cells engineered to express antigen-specific CARs. Other intracellular domains include the intracellular portion of CD28 and CD3-zeta. The extracellular portion of FRa-CAR *Sensor-Receptor* comprises the CD8 segment proximal to the cell membrane as a hinge to facilitate the formation of immune synapse; and the FRa-specific *Sensor* at the distal end with variable domains from heavy (V_H) and light (V_L) chain

of the anti-FRa antibody (MORAb-003) is optimized for human expression. The *Sensor* portion determines the specificity for antigen-presenting cells. The FRa-CAR2 (with 5'-V_H-V_L-3') and FRa-CAR1 (with 5'-V_L-V_H-3') were compared to determine the favorable positions of V_H and V_L relative to each other. Based on this comparison, the 5'-V_H-V_L-3' orientation was identified as the preferred orientation for the *Sensor* design. The transmembrane portion that connects the extracellular and intracellular portions is from the CD28 and extends as the intracellular co-stimulatory domain.

iii) ***Figure S3*** **(Selection of an efficient *Secretor*).** Plasmid with optimally configured *Actuator* and FRa-specific *Sensor-Receptor* sequences (FRa-CAR3$_{NFAT\text{-}RE6X}$→Nluc-2A-GFP) was modified in five different ways with different signal peptides as *Secretor* for the *Effector*. The signal peptides used were from human serum albumin (HSA), interleukin (IL)-6, IL-2, Gaussia luciferase (Gluc) or interferon (IFN) alpha-2, as per **Fig. S3(i)**. The Jurkat Clone E6-1 suspension cell line was engineered with the lentivirus particles prepared from these plasmids. The five *T-cell Biofactories* (per **Fig. 1B**) and a control (per **Fig. 1B** but without any *Secretor*) were biologically stimulated (using the parental OVCAR3 (FRa^+) cells or A2780cis (FRa^{neg}) cells) for 6 hrs (See *Methods Section* "Signal Peptide Experiment and Co-culture" for the complete procedure). The S/N for each *T-cell Biofactory* was determined by calculating the ratio of means of Nluc activity from the *T-cell Biofactory* stimulated by parental OVCAR3 (FRa^+) cells to that from stimulated by parental A2780cis (FRa^{neg}) cells (**Fig. S3(ii)**). The error bars extend 1 SD above the mean and can also be considered as one half-width of an 86% confidence interval for that mean. Based on these data, IFNa2 was selected as the *Secretor* for use in generating the *T-cell Biofactory* as shown in **Fig. 1A**.

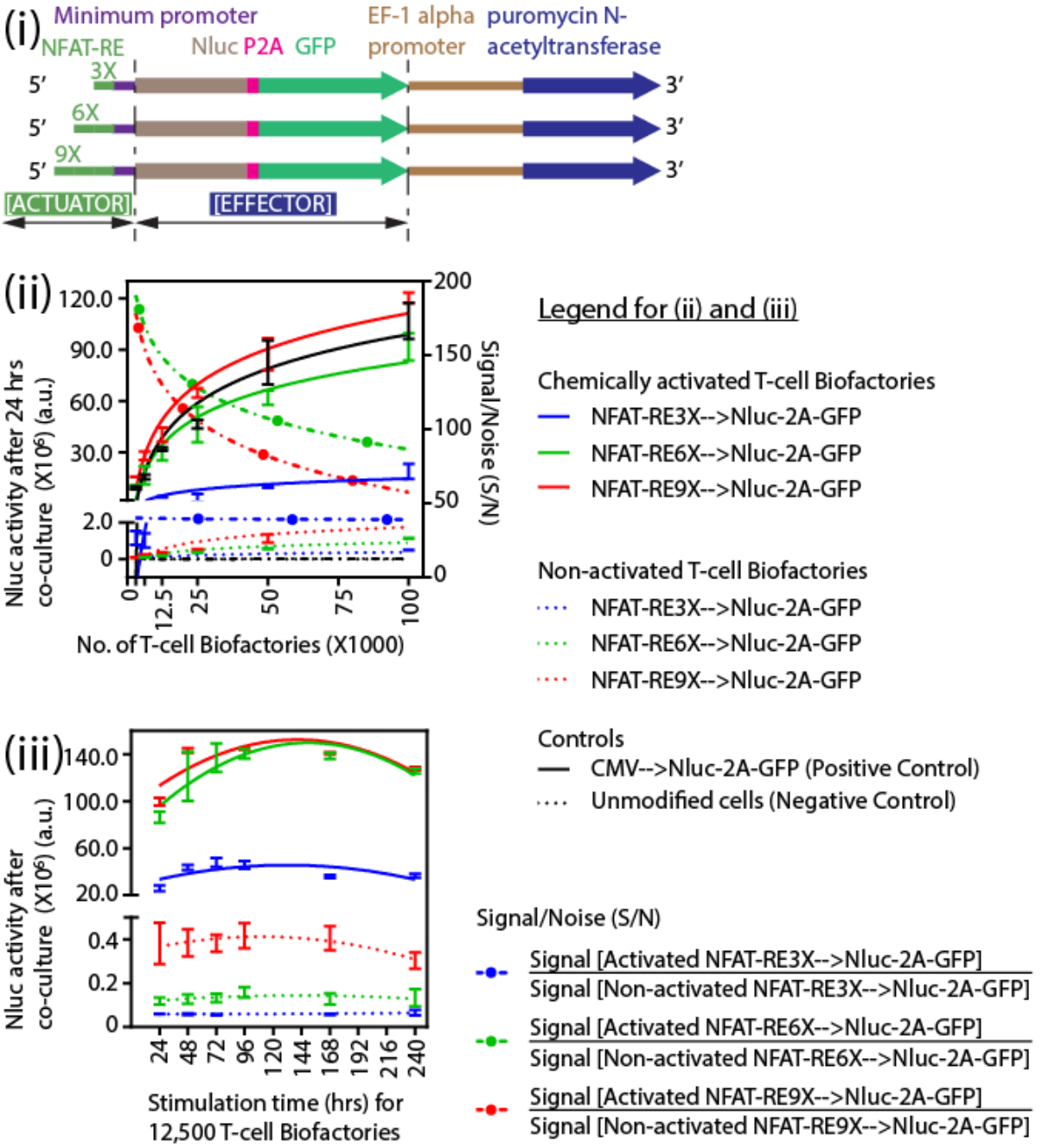

Figure S1. ***Design and selection of the Actuator.*** (i) Schematic for the *T-cell Biofactories* insert encoding different variations of the *Actuator*. The Nluc activity for activated and non-activated forms of three differently engineered *T-cell Biofactories* [per **Fig. S1(i)** (NFAT-RE3X; NFAT-RE6X; NFAT-RE9X); and one control with CMV regulated reporters] was measured w.r.t. the (ii) number of T-cell Biofactories, (iii) stimulation time. [n=3 for (ii); n=4 for (iii), error bar indicates ± 1 SD)].

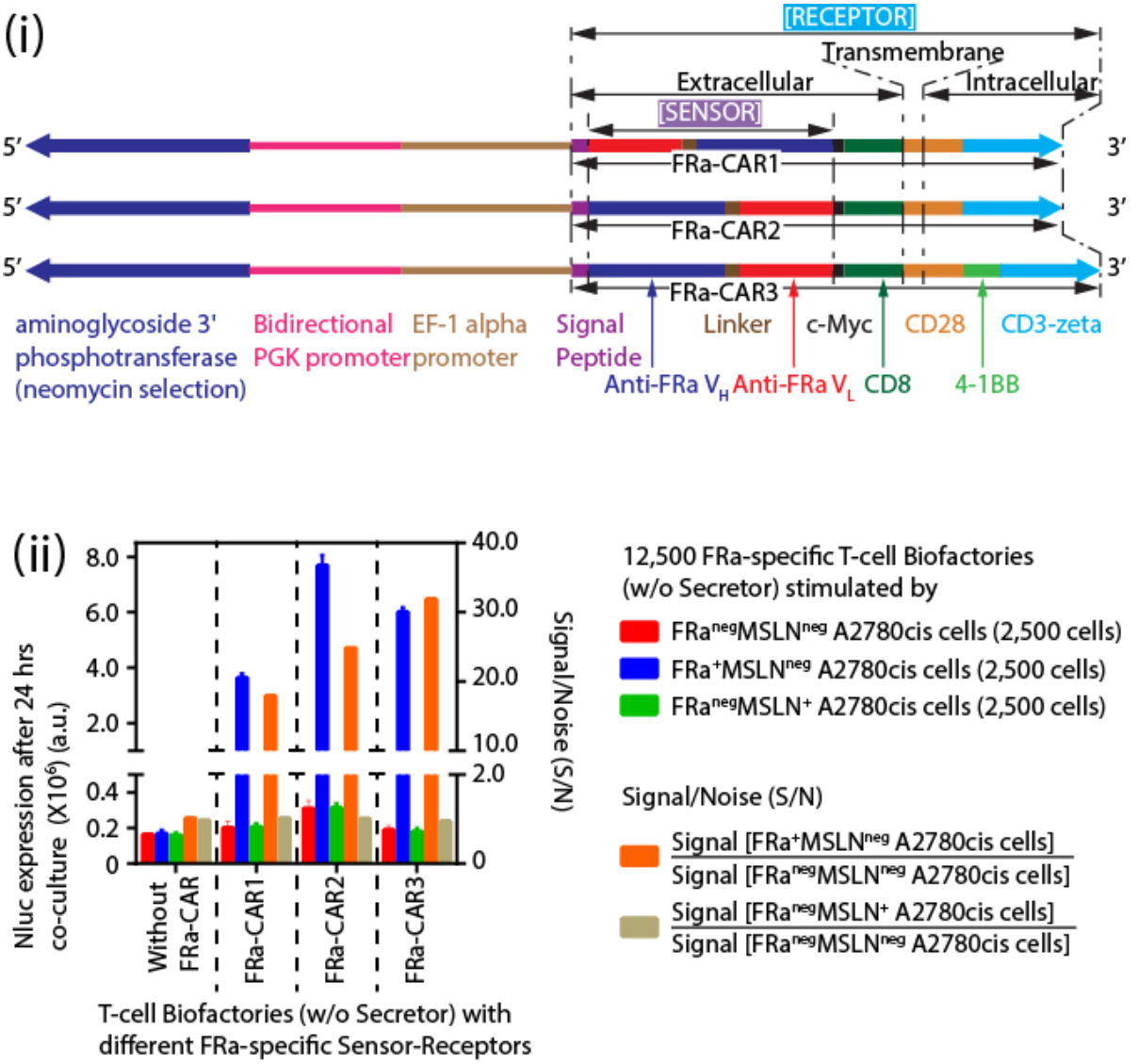

Figure S2. ***Design and selection of the Sensor-Receptor.*** (i) Schematic for the *T-cell Biofactories* insert encoding different variations of the *Sensor-Receptor*. (ii) The Nluc activity (n=4, error bar indicates ± 1 SD) for activated and non-activated forms of four differently engineered *T-cell Biofactories* [per **Fig. S2(i)** (FRa-CAR1; FRa-CAR2; FRa-CAR3); and one control without the FRa-CAR *Sensor-Receptor* domain] when stimulated by three different target/non-target cells ($FRa^{neg}MSLN^{neg}$ A2780cis; $FRa^{+}MSLN^{neg}$ A2780cis; $FRa^{neg}MSLN^{+}$ A2780cis).

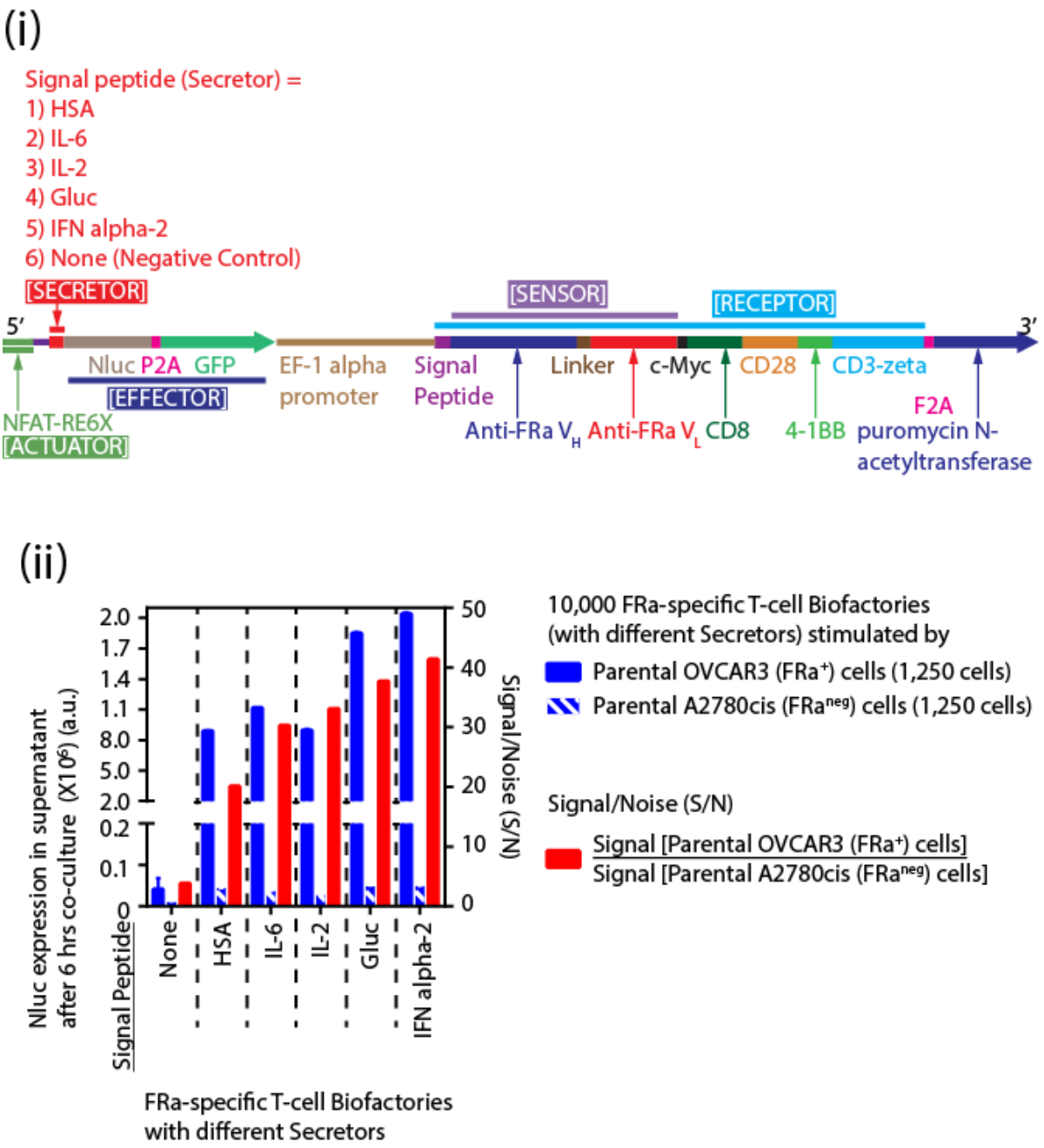

Figure S3. ***Selection of an efficient Secretor.*** (i) Schematic for the *T-cell Biofactories* insert with different variations of the *Secretor*. (ii) The Nluc activity (n=4, error bar indicates ± 1 SD) for activated and non-activated forms of six differently engineered *T-cell Biofactories* [per **Fig. S3(i)** (five with signal peptides from HSA, IL-6, IL-2, Gluc, IFN alpha-2); and one control without any signal peptide *Secretor* domain] when stimulated by two different target/non-target cells (parental OVCAR3 (FRa^{+}) cells A2780cis (FRa^{neg}) cells).

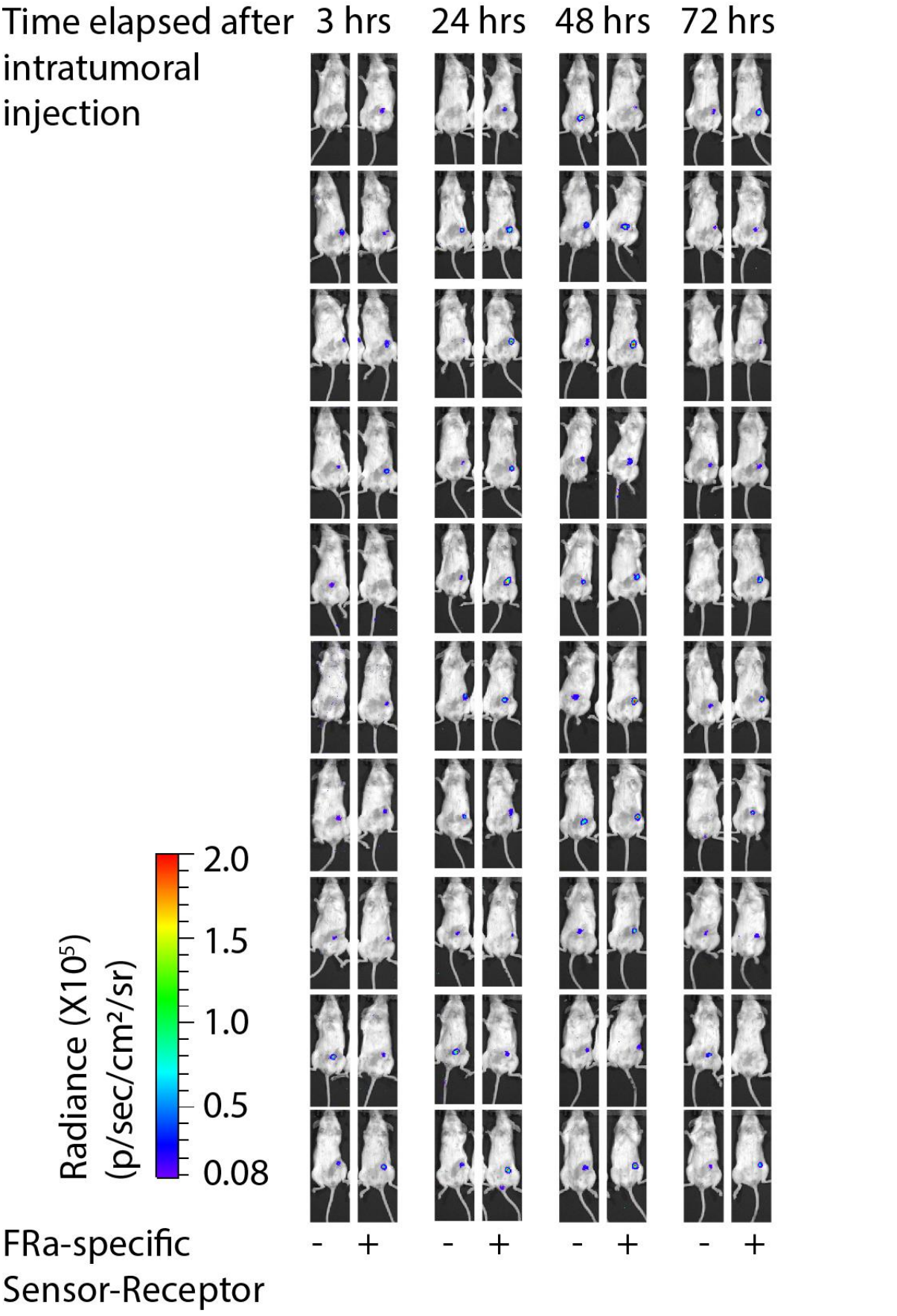

Figure S4. ***In vivo validation (all mice) of the T-cell Biofactory.*** Data for the longitudinal bioluminescent imaging of all mice (n=10) at the indicated time points. (See *Experimental Methods Section* "*In vivo* study/ Bioluminescent imaging" for the complete procedure).