Supporting Information for

Abnormal Scar Diagnosis with Topical Spherical Nucleic Acids

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Materials and Methods
Methods

All materials, except those specifically mentioned, were purchased from Sigma-Aldrich. NanoFlare RNA detection probes (SmartFlare®) were purchased from Merck Millipore. Primers for RT-qPCR were purchased from AIT Biotech (Singapore). Dulbecco's modified eagle medium (DMEM) high glucose with L-glutamine was purchased from Lonza (Singapore). Fetal bovine serum (FBS), trypsin–EDTA (0.05%), and penicillin–streptomycin (10,000 U/ml) were purchased from Gibco. Vybrant DiI Cell-Labeling Solution, Geltrex LDEV-Free Membrane Matrix and NucBlue Live ReadyProbes Reagent were purchased from Thermo Fisher Scientific. DNase/RNase-free distilled water and TRIzol reagent were purchased from Invitrogen. M-MLV reverse transcriptase was purchased from Promega. iQ SYBRGreen Supermix was purchased from Bio-Rad. All animal experiments performed have been approved by the Institutional Animal Care and Use Committees (IACUCs: #BN16098).

Cell Culture: Normal dermal fibroblasts (NDFs), hypertrophic scar-derived fibroblasts (HSFs), normal fibroblasts adjacent to hypertrophic scars – peri-hypertrophic scar fibroblasts (PHSF), keloid scar-derived fibroblasts (KSF), and normal fibroblasts adjacent to keloid scars – peri-keloid scar fibroblasts (PKSF) were obtained from CellResearch Corporation Pte Ltd, Singapore. Cells were cultured in high-glucose DMEM containing 4 mM L-glutamine and supplemented with 10% FBS (37 °C, 5% CO_2). Culture medium was replaced every 2–3 days. For experimental purposes, cells were used between passage 3 to 10.

Cell Labeling with SNAs: 1µl of stock SNAs solution (100 nM) were diluted into 20 µl of 1 × Phosphate-buffered saline without calcium and magnesium (PBS, Gibco), before being added into 1ml of complete culture medium with 10% FBS. Cells were then incubated with this labeling solution for 18 hours before being washed with ice-cold PBS. All SNAs used in this study can be found in Table 1.

Fluorescence Imaging: Fluorescence images were taken using an LX71 inverted fluorescence microscope (Olympus) equipped with a Retiga-2000R CCD camera. All images were captured with an exposure time of 750ms and a gain number of 5, and processed to remove background through ImageJ software. Image analysis was also performed with ImageJ to quantify cellular fluorescence intensity post-NanoFlare labeling.

For confocal imaging, NanoFlare-labeled cells were washed thrice with pre-chilled PBS (4 °C) and resuspended at 10^6 cells/ml density, prior to membrane dye staining (5 µM Vibrant DiI) for 5 minutes at 37 °C. After washing 3× with cell culture medium, cells were re-seeded on fibronectin-

coated cover slips for 20 minutes. Then cells were fixed with chilled 4% paraformaldehyde in PBS for 10 minutes on ice. Later, NucBlue® Live cell stain (i.e. Hoechst 33342) was used to stain the nucleus before mounting onto microscope slides for visualization with confocal microscope LSM710 (Zeiss). DiI was depicted using green color and NucBlue® blue color. Cell imaging experiments were performed in triplicate and repeated unless otherwise stated.

Flow Cytometry: Approximately 1 million cells were washed in PBS and then re-suspended in cold FBS until the flow cytometry analysis using the LSR Fortessa™ X-20 Flow cytometer (Becton Dickinson). Unlabeled cells were used as gating control samples to evaluate percentage and average intensity of labeled cells. The gating was determined by setting the threshold at a given fluorescence intensity which minimizes the unlabeled cell population but maximizes the corresponding number of labeled cells. Flow cytometry experiments were performed in triplicate and repeated unless otherwise stated.

High Throughput Evaluation of Anti-fibrotic Compounds: 1×10^4 HS cells were seeded in each well in the 96 well-plate. Upon reaching 85-90% confluency, the medium was replaced with low serum medium (0.5% FBS). 12 hours later, TGFβ1 (10ng/ml), RepSox (25μM), decorin (100nM), Thiazovivin (500nM), simvastatin (60μM), or perfinidone (50μg/ml) were added to wells. After 48 hrs treatment, the solution was replaced with SNA-containing complete culture medium for another 18 hrs. Finally, cells were rinsed thrice with ice-cold PBS, nuclei-stained with NucBlue® Live cell stain, and analyzed with Synergy™ H4 (BioTek). 5x5 area-scanning of respective fluorescence channel was then done to evaluate the effects of these small molecules on CTGF and TGFβ1 expression. High-throughput screening experiments were performed in triplicate and repeated unless otherwise stated.

Small Interfering RNA (siRNA) Transfection: Cells near confluency (90-95%) were transfected using Lipofectamine® 2000 (ThermoFisher Scientific, Singapore) at a final concentration of 60 nM (based on manufacturer's protocol). Briefly, 1 μL Lipofect solution was prepared with 200 μL transfection medium (DMEM + 1% FBS). 3 selected siRNA duplexes (sense 5'-GACAUCUAUGCAAUGGGCUUAGUAU-3', 5'-GCAUCUCACUCAUGUUGAUGGUCUA-3', 5'-AGUAAGACAUGAUUCAGCCACAGAU-3') which covered different regions of the human TGFβRI mRNA[23] were applied in low serum medium (DMEM + 1% FBS) for 48hr (60 nM concentration). Silencer® Select Negative Control siRNA (Thermofisher Scientific, Ambion, Singapore) catalog: #4390843 was chosen as the noncoding (Scrambled) negative control transfection.

RT-qPCR Analysis: 1×10^6 untreated, RepSox-treated or TGF-β1-treated cells were resuspended and lysed with TRIzol reagent. Following RNA extraction of the samples, cDNA conversion was performed with reverse transcription kit M-MLV Rnase H(-) Mutant. Primer sequences for CTGF and GAPDH mRNA are listed in Table 1. Then, C_T values of each gene were obtained through quantitative PCR (CFX ConnectTM PCR System, Biorad). Finally, $2^{-\Delta\Delta CT}$ formula was adopted to evaluate CTGF-fold expressions between the sample groups (normalized against untreated NDF). RT-qPCR analysis was performed in triplicate and repeated unless otherwise stated.

Immunostaining: 1×10^5 cells were seeded on 6 well-plates. Upon reaching 85-90% confluency, cells were rinsed with PBS and fixed with chilled 4% paraformaldehyde in PBS for 10 minutes on ice. Cells were then stained with Monoclonal Anti-Actin, a-Smooth Muscle-Cy3 clone 1A4 (Sigma Aldrich) for 18 hrs at 4˚C, at a concentration of 6 µg/ml. After rinsing thrice with cold PBS, NucBlue® Live cell stain was used to stain the nucleus before imaging. Immunostaining and imaging was performed in triplicate and repeated.

IVIS® Spectrum In vivo imaging system imaging of subcutaneous-injected, NanoFlare-labeled human fibroblasts: Except as otherwise stated, ~2×10^6 NDF and HSF were incubated for 4 hrs with NanoFlare-labeling medium (SCRM/CTGF-Cy5), as previously described. Cells were then resuspended in 100 µl of medium and 100 µl of Matrigel in pre-chilled tubes. The chilled mixture (4°C) was then injected subcutaneously into the flanks of nude mice with pre-chilled syringes and 30G-needles. Mice were kept anesthetized for an additional 10 mins to allow Matrigel gelation. 24 hrs later, mice were taken for fluorescence imaging with IVIS® Spectrum CT (PerkinElmer, Singapore Pte Ltd). The field of view, which covered the entire mouse (13.6 cm), height distance, and optical gain were kept constant throughout the measurement. At the end, the averaged Cy5 fluorescence intensity from each region-of-interest was recorded. IVIS imaging was performed on at least triplicate mouse subjects and repeated unless otherwise stated.

Penetration of topically applied NanoFlares in mice: NanoFlares were topically applied based on previously reported protocols with minor modifications.[15] Briefly, 50nM NanoFlares (SCRM, GAPDH Cy5) in PBS: Aquaphor® (Beiersdorf) (1:1) was applied to the dorsal skin of nude mice for up to 48 hrs and thoroughly cleaned with PBS prior to IVIS imaging, as described above. Subsequently, skin samples were harvested and cryo-sectioned for fluorescent imaging.

**Table 1: NanoFlare and primer information**

NanoFlare	Product number
CTGF Human (Cy3 & Cy5) NanoFlare	5'-CCAACTGCCTGGTCCAGACCACAGAGT-3' (sequence obtained from Merck-Millipore) SF-1984
Scrambled (Cy5) NanoFlare *	SF-102
Uptake (Cy5) NanoFlare *	SF-137
GAPDH Human Cy3*, Mouse Cy5 NanoFlare *	SF-126, SF-138
Primers	**Primer sequences (5'->3')**
CTGF primer-forward	CAGCATGGACGTTCGTCTG
CTGF primer-backward	AACCACGGTTTGGTCCTTGG
GAPDH primer-forward	GAAGGTGAAGGTCGGAGT
GAPDH primer-backward	GAAGATGGTGATGGGATTTC
TGFβ1 primer-forward	GGCCAGATCCTGTCCAAGC
TGFβ1 primer-backward	GTGGGTTTCCACCATTAGCAC

(* Exact NanoFlare gene recognition sequences are proprietary information of Merck Millipore and are not freely available)

Supporting Figures: S1 – S17
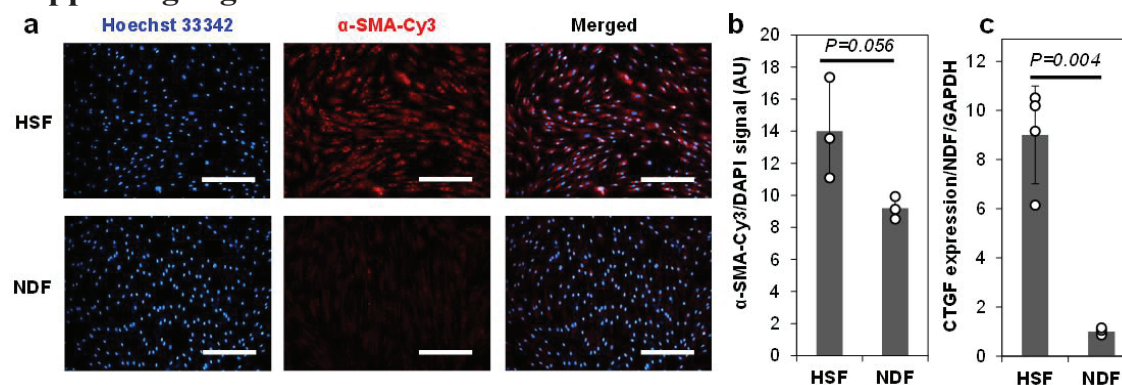
Figure S1: Difference between fibroblasts derived from HSF and NSF. **a**) Immunostaining of α-SMA protein expression in NDF and HSF. **b**) Quantification of α-SMA-Cy3 signal normalized against Hoechst 33342 signal (N=3). **c**) qPCR analysis of CTGF mRNA expression in HSF and NDF cells normalized with GAPDH expression in NDF (N=4). Values are mean ± SD.
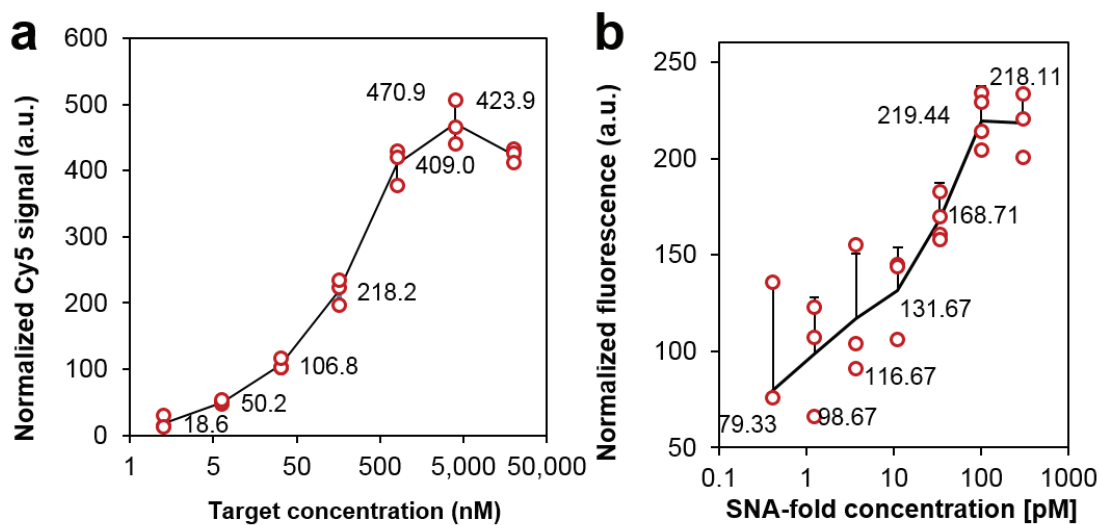

Figure S2: **Optimization of oligonucleotide target (CTGF mRNA surrogate) concentration and NanoFlares concentration**. (**a**) the fluorescence intensity of 0.1 nM concentration of CTGF NanoFlares when the target concentration was varied from 1.28 to 20,000 nM (N=3). (**b**) The fluorescence intensity of NanoFlares solution following the addition of the 800 nM target sequence normalized by the signal prior to oligonucleotide addition (N≥3). Values are mean ± SD.
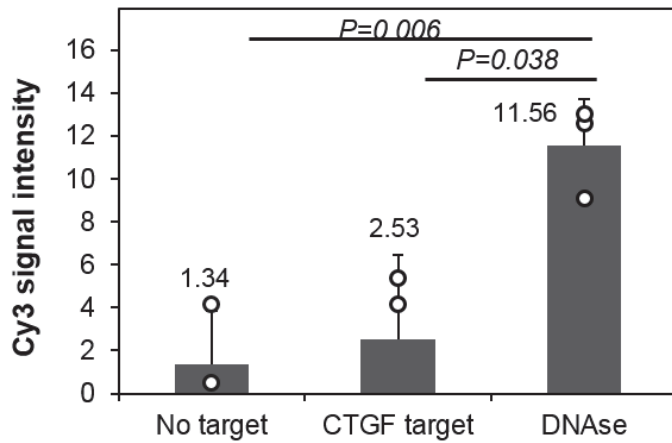
Figure S3: Cy3 fluorescence signal from GAPDH NanoFlares in the mixture of GAPDH NanoFlares and CTGF NanoFlares before and after the addition of CTGF target and DNase (N=3). Values are mean ± SD.
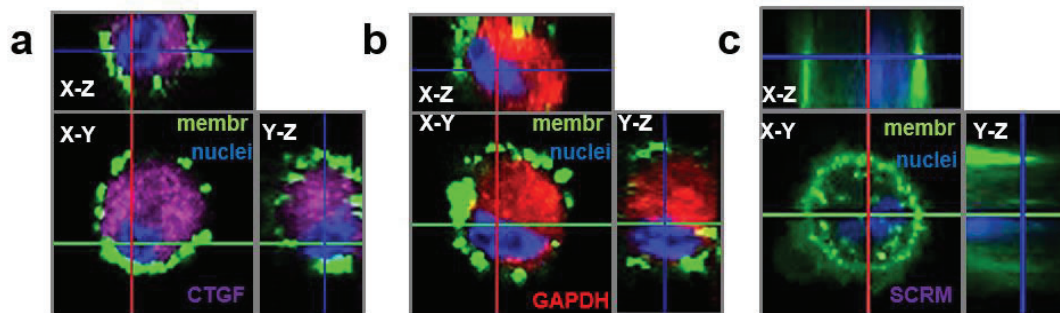

Figure S4: Confocal images of HSF labeled with (**a**) CTGF-Cy5 (magenta) NanoFlares, (**b**) GAPDH-Cy3 (red) NanoFlares and (**c**) scrambled (SCRM)-Cy5 (magenta) NanoFlares. All cells were post-labeled with DiI (green) to define the cell membrane (membr) and Hoechst 33342 (blue) for the nuclei. The individual planes are defined as indicated : X-Y, X-Z, Y-Z plane views.
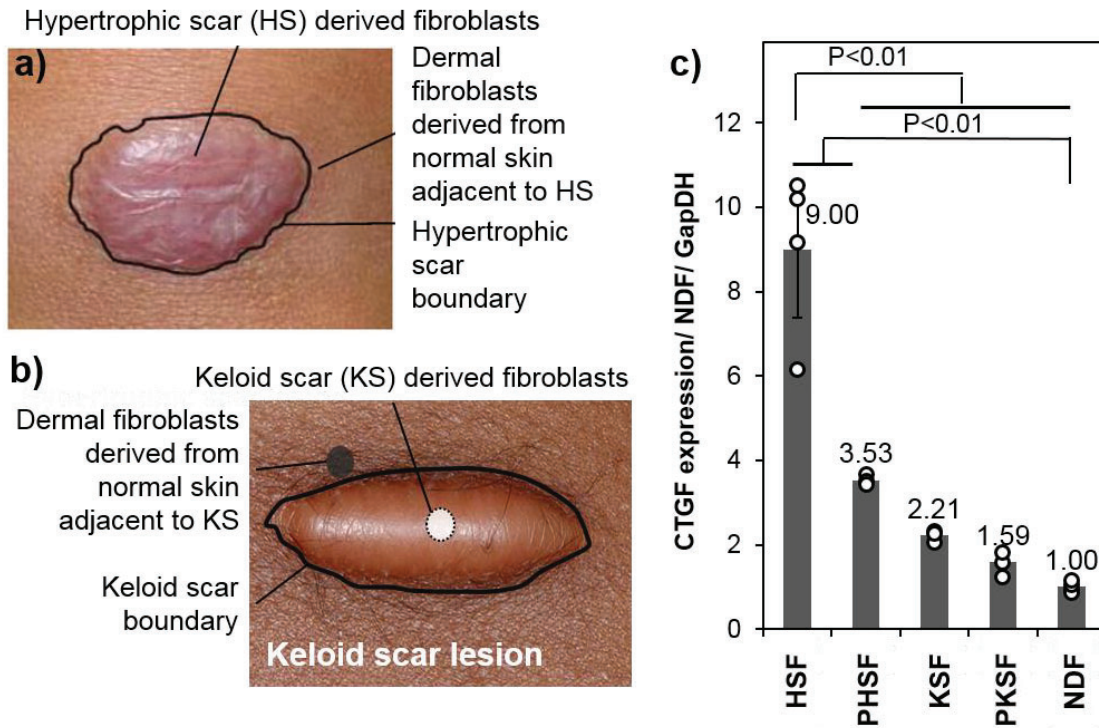
Figure S5: Location of biopsies for fibroblast cultures and CTGF mRNA measurement. (a) Representative photograph delineating the location from which tissue was taken for cultured fibroblasts from hypertrophic scars (a) and keloids (b). Hypertrophic scar fibroblasts, keloid scar fibroblasts were harvested from the indicated region within the respective lesions. Near-hypertrophic scar fibroblasts (peri-hypertrophic scar fibroblasts, PHSF) and near-keloid scar fibroblasts (peri-keloid scar fibroblasts, PKSF) are indicated by the shaded biopsied area. (c) CTGF expression of dermal fibroblasts derived from abnormal scars (NDF, PHSF, PKSF, HSF and KSF), normalized to NDF expression and GAPDH gene expression (N=4). Values are mean ± SD.
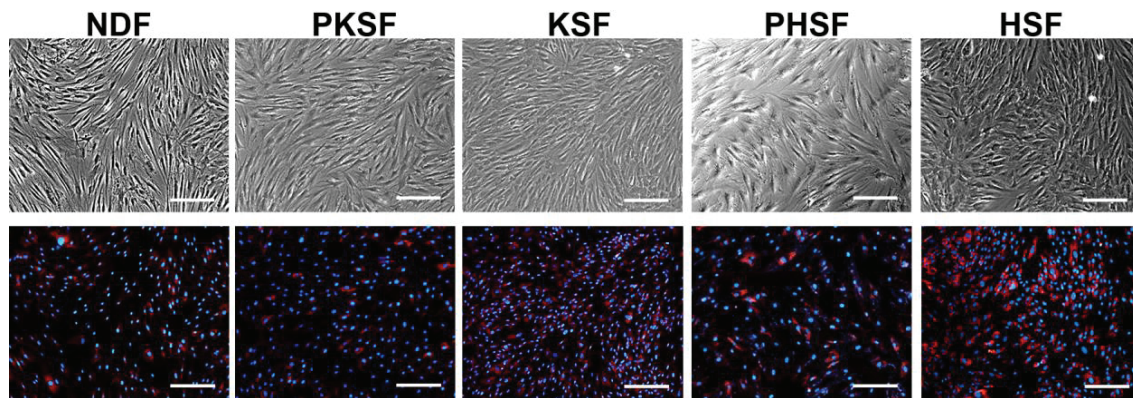
Figure S6. Representative phase contrast and merged fluorescence images of NDF, PKSF, KSF, PHSF, and HSF after being labeled with CTGF NanoFlare (red fluorescence false color for CTGF Cy5 signal) and Hoechst (H) 33342 (blue color). Scale bar: 100 µm.
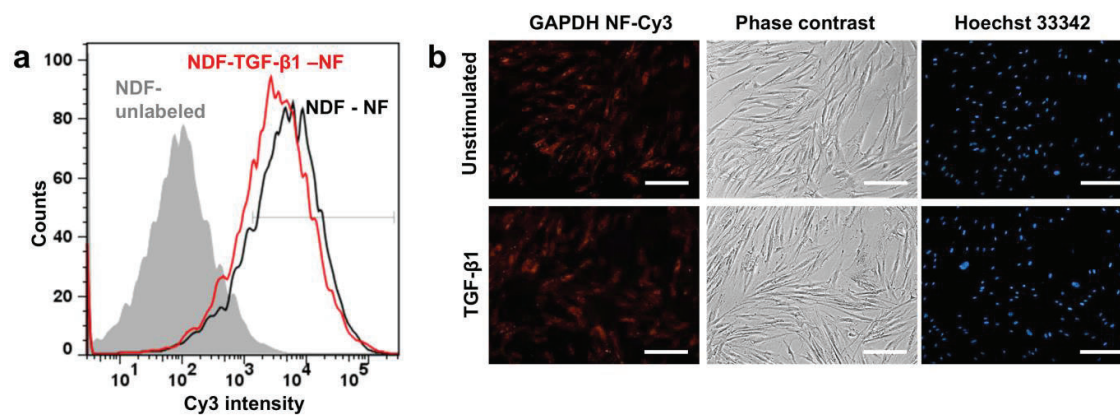

Figure S7: **GAPDH NanoFlares (NF) to analyze NDFs pretreated with TGF-β1**. **a**) Flow cytometry histogram of NDF without GAPDH NF, NDF with GAPDH NF, and NDF treated with TGF-β1 and GAPDH NF; **b**) Representative fluorescence images of NDF with GAPDH NF, and NDF treated with TGF-β1 and GAPDH NF.
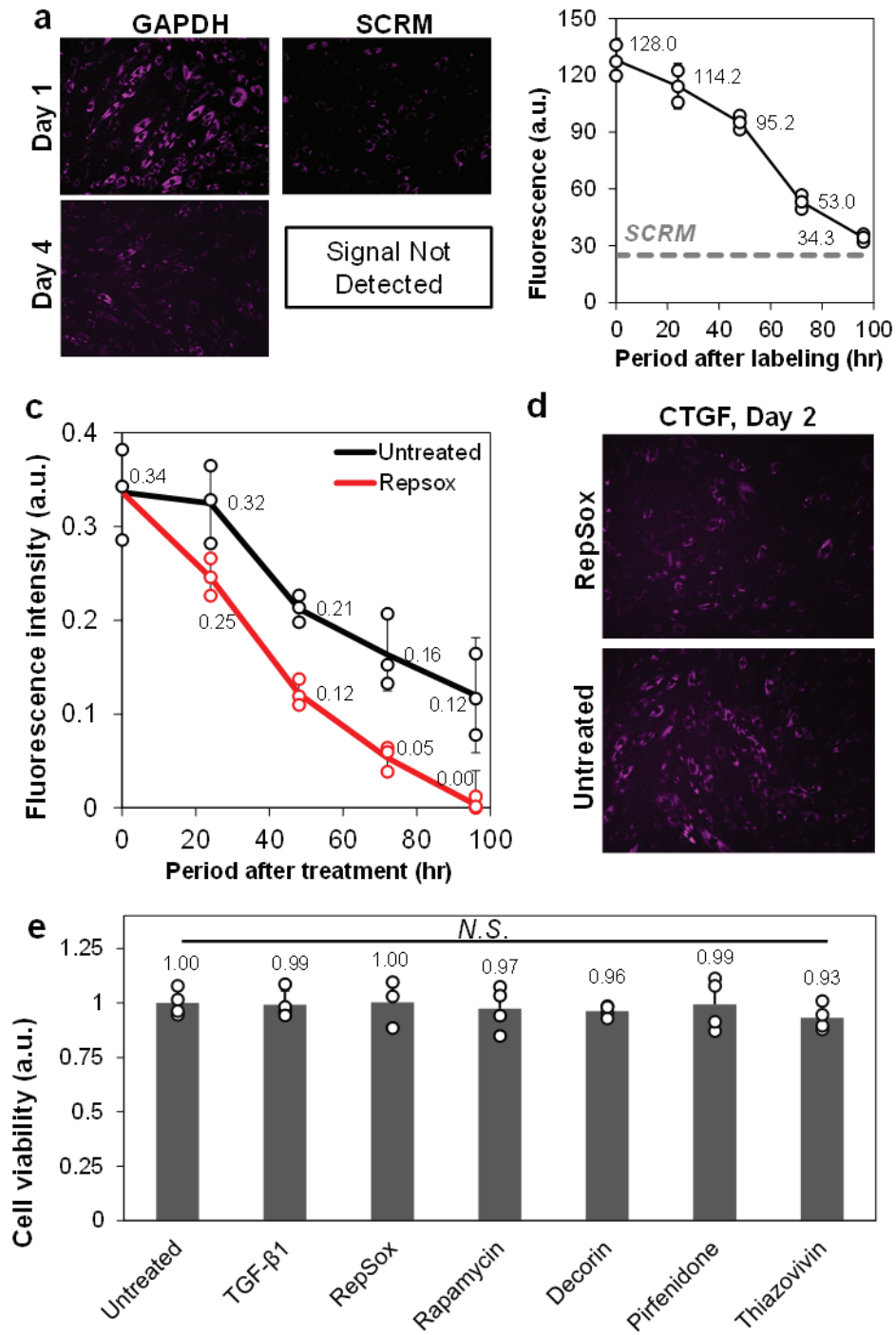
Figure S8. NanoFlare expression kinetics and HSF drug treatment viability. a) Representative images of GAPDH, SCRM NanoFlares (Cy5, purple) at day 1, 4 (signal undetected for SCRM day 4). b) Signal expression kinetics of GAPDH NanoFlares over a period of 4 days after labeling. c) Fluorescence expression kinetics for untreated and RepSox treated HSF for a period of 4 days (96 hrs, N=3). d) Representative images (purple: Cy5) at day 2 (48 hrs) for RepSox and untreated HSFs. e) HSF viability after drug treatment (N=4). Esterase activity (fluorescein diacetate) for each treatment normalized by cell number (Hoechst 33342 labeling). Values are mean ± SD.
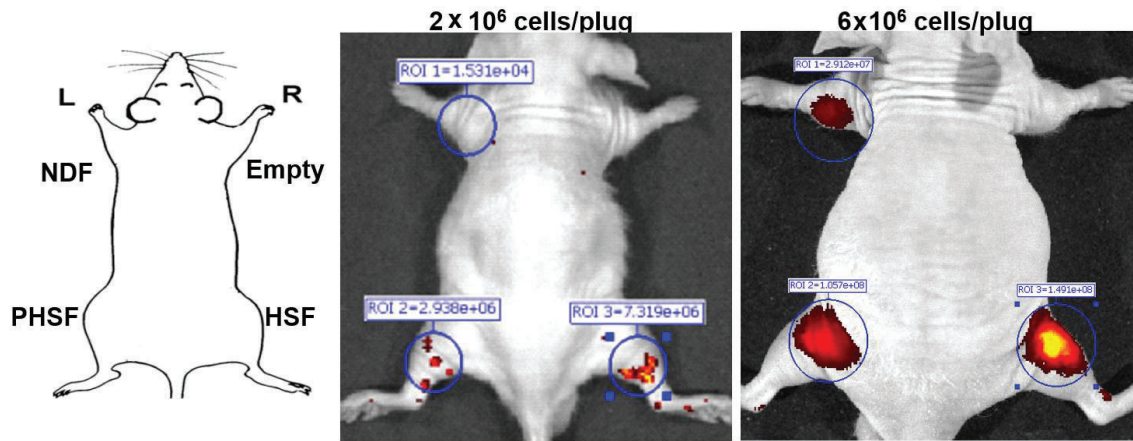
Figure S9. Optimization of cell concentration (2, 6×10^6 cells/100 µl matrigel) in the matrigel plug via IVIS signal. NDF, PHSF, HSF and empty gels were injected into both fore and hind upper limbs.
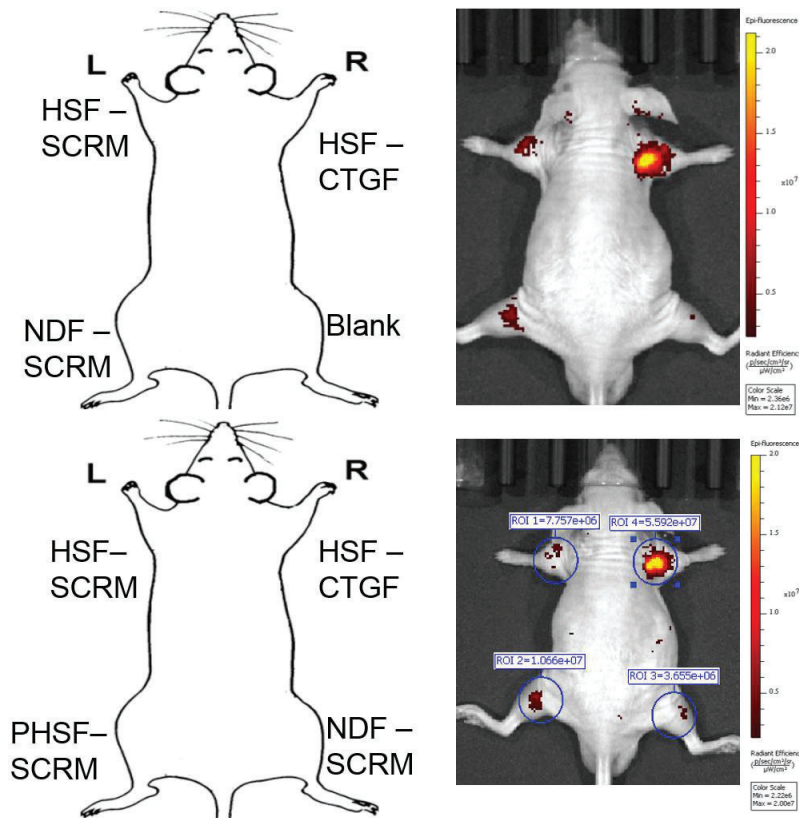
Figure S10. Ascertaining non-specific NanoFlare signal by comparing SCRM, blank gels with HSF-CTGF (positive control). Schematic (Top left), representative image (Top right). Schematic (Bottom Left) and representative image (Bottom right) with SCRM groups (HSF, PHSF, NDF) compared to HSF-CTGF (positive control).
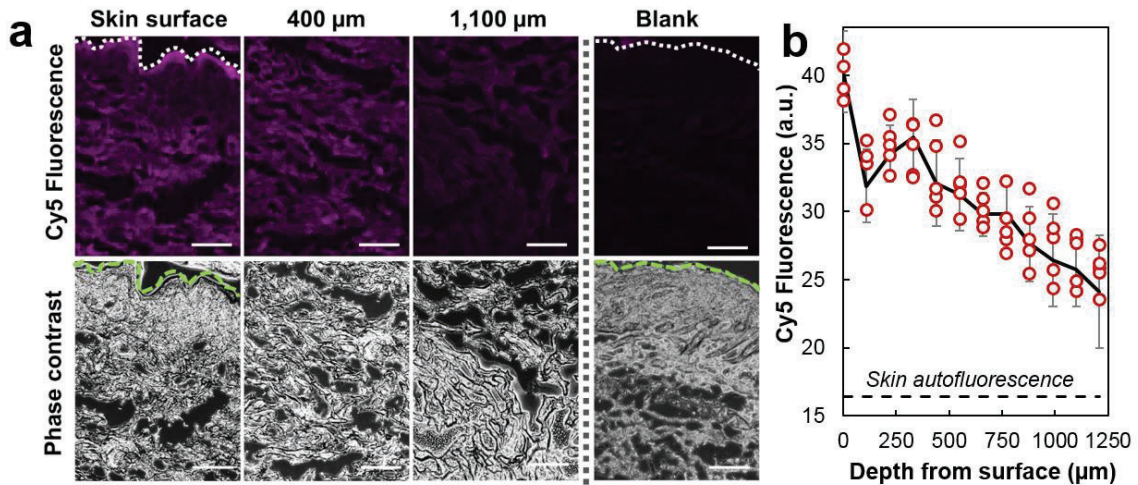
Figure S11. **Cy5 NanoFlare signal distribution throughout skin cross section**. a) Representative fluorescence (purple) and phase contrast images of "*Uptake Control-Cy5*" NanoFlares topically applied to skin surface compared to unmodified skin (blank). b) Relative quantity of NanoFlares (obtained from normalized Cy5 signal via image analysis) at the indicated skin depth (N=4). Scale bar represents 100μm.
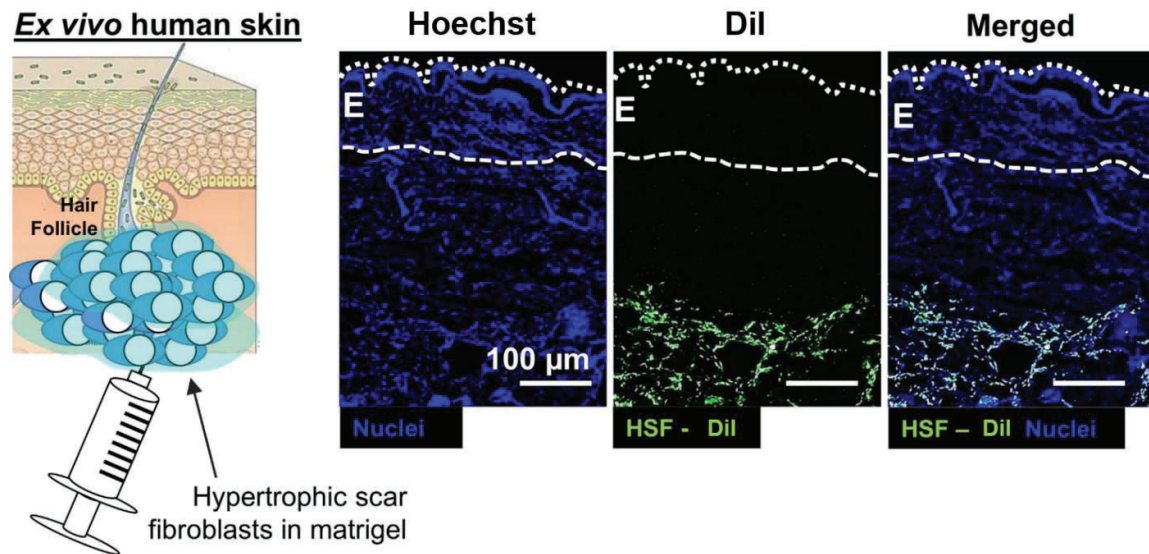
Figure S12. *Ex vivo* human skin abnormal scar models. Schematic showing HSF-matrigel mixture injected into the dermis region of *ex vivo* human skin sections (left most). Representative blue fluorescence (cell nuclei), fluorescence (DiI labeled HSF cells) and corresponding merged images of skin sections. The epidermis region is demarcated by the (-----) boundaries (E). Scale bar represents 100μm.
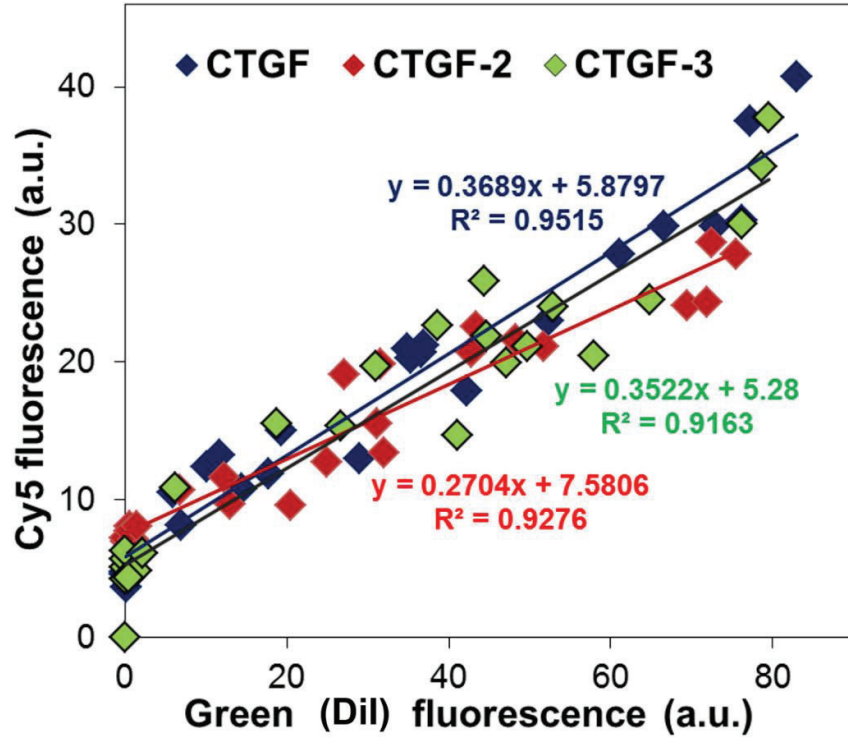
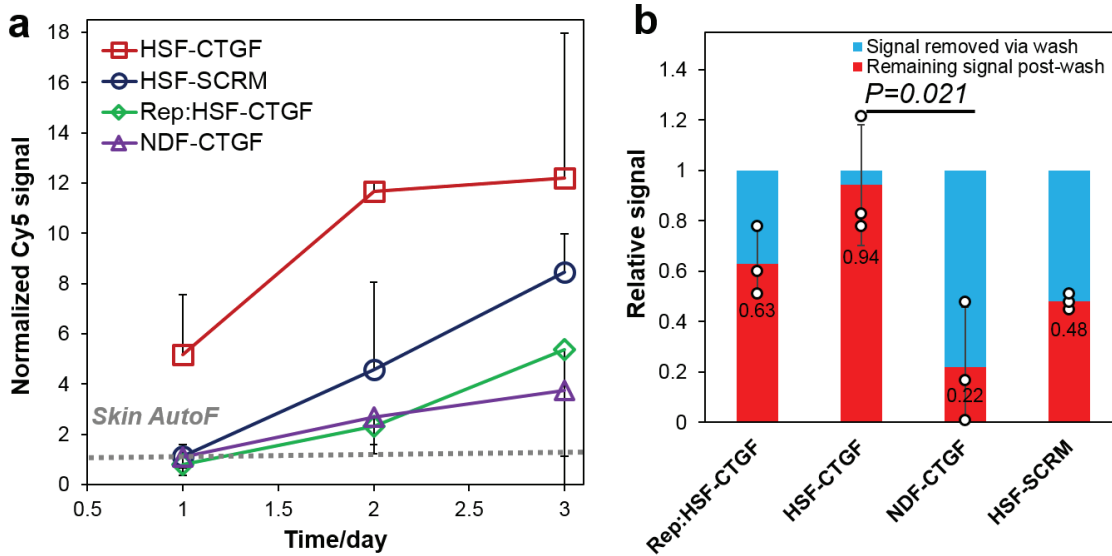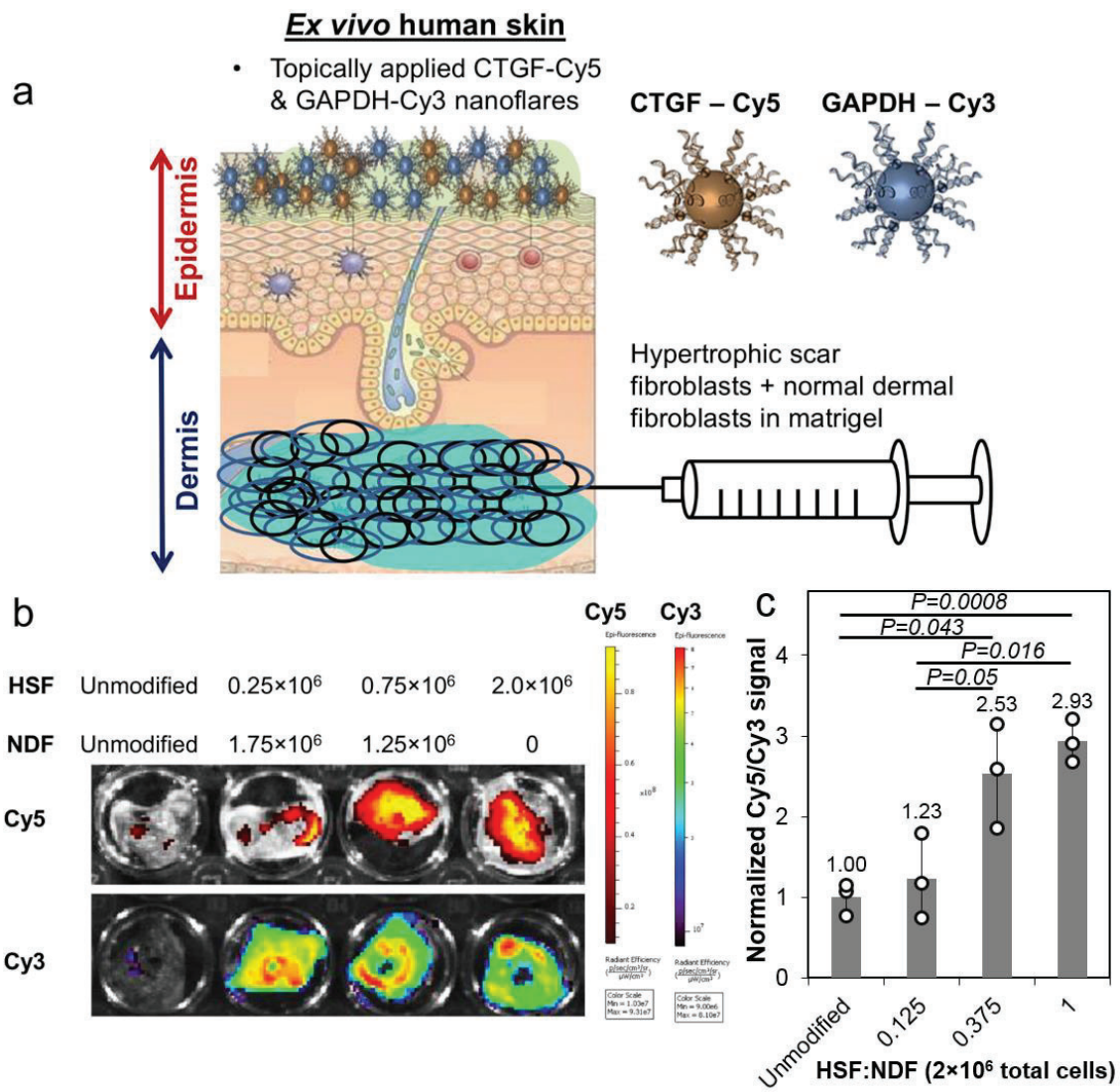**Figure S13**. Three independent experiments to demonstrate the CTGF NanoFlare specificity.


Figure S14. **NanoFlare signal variation with time and pre/post-washing**. a) Normalized signal with respect to post nanoflare application time (N=3). b) Relative signal strength before and after sample wash (N=3).

Figure S15. **Non-invasive quantification of abnormal scarring biomarkers with topically applied nanoflares.** a) *Ex vivo* skin samples were injected through the dermal side with various ratios of unlabeled HSFs and NDFs are topically treated with a dual nanoflare mixture (CTGF-Cy5, GAPDH-Cy3) to non-invasively evaluate the extent of abnormal scarring by the relative Cy5/Cy3 signal ratio. The respective Cy3 and Cy5 scales are shown (right). b) Representative IVIS images and c) fluorescence signal quantification of ex vivo human skin containing the indicated combinations of HSFs and NDFs (total: $2×10^6$ cells per skin sample, N=3). Values are mean±SD.
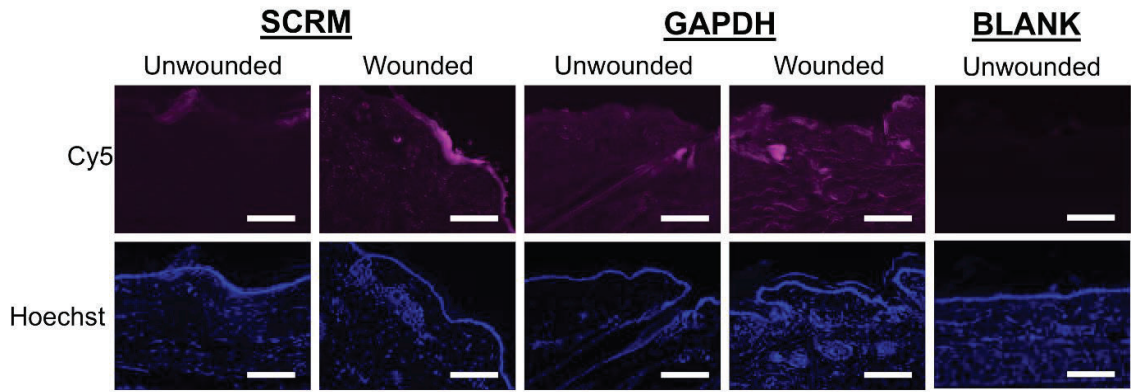
Figure S16: Tissue sections of unwounded and wounded rabbit ear skin following topical application of SCRM and GAPDH nanoflares. Cy5 and Hoechst 33342 fluorescence channels. Scale bar represents 100μm
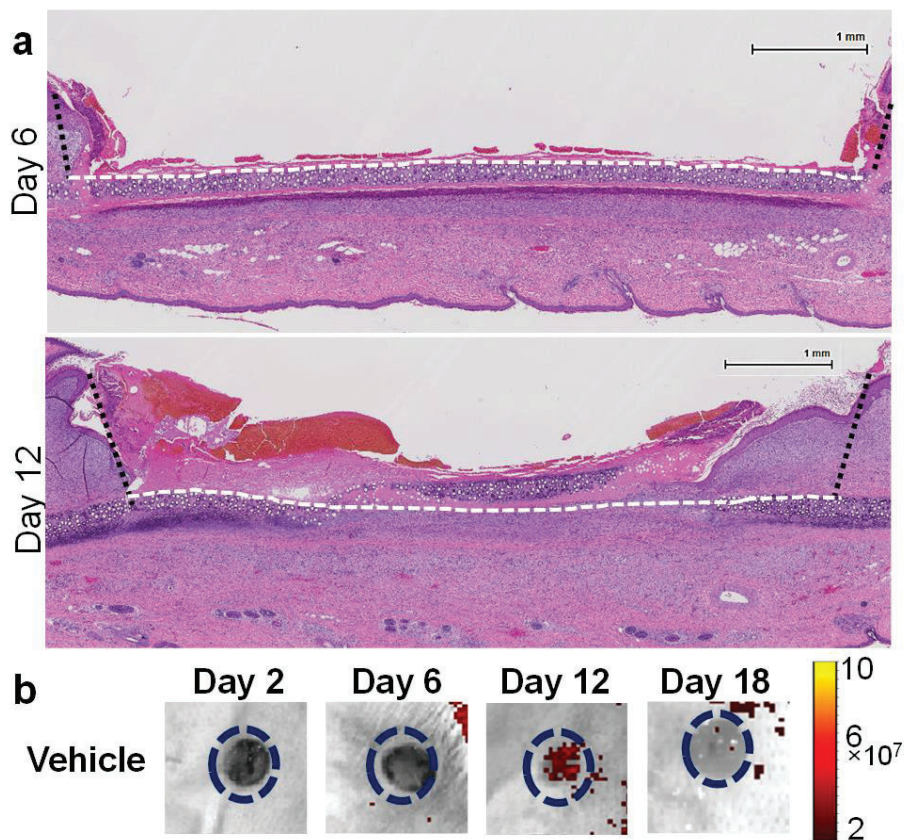
Figure S17: Supporting wound histology and IVIS images. a) H&E stained images of rabbit ear wounds harvested on day 6 and 12 (scale bar: 1mm). b) Representative IVIS images of 'vehicle' application on days 2, 6, 12, 18. The ring demarcates the generated wound (7 mm diameter).