Supporting Information for:

Aurophilically cross-linked "dynamic" hydrogels mimicking healthy synovial fluid properties

Pablo Casuso,[a] Adrián Pérez-San Vicente,[a] Haizea Iribar,[b] Araika Gutiérrez-Rivera,[b] Ander Izeta,[b] Iraida Loinaz,[a] Germán Cabañero,[a] Hans-Jürgen Grande,[a] Ibon Odriozola[a] and Damien Dupin[a]**

[a] *New Materials Division, IK4-CIDETEC Research Centre, Paseo Miramón 196, Donostia-San Sebastián 20009, Spain*

[b] *Tissue Engineering Laboratory, Bioengineering Area, Instituto Biodonostia, Hospital Universitario Donostia, Paseo Dr. Begiristain s/n, 20014 San Sebastián, Spain.*

*To whom correspondence should be addressed: ddupin@cidetec.es

Experimental procedures

Materials

PEG-dithiol (M_n = 3,400 g mol^{-1}), $HAuCl_4$ (≥ 49 % Au Basis), *2,2'*-thiodiethanol (≥ 99 %) and 5,5'-dithiobis-(2-nitrobenzoic acid) [DTNB] (≥ 98 %) were used as received. HCl (1 M) and NaOH (1 M) were purchased from Scharlau and used as received.

Methods

Gold(I) solution preparation[1]

0.1 M gold (I) solution was prepared adding dropwise a solution of 2,2´-thiodiethanol (20 µL in 480 µL in water) to a solution of $HAuCl_4.3H_2O$ 0.2 M (0.5 mL). Both solutions were maintained in an ice-bath before the adding and were vigorously shaken with a vortex after each drop was added. All the process was maintained at 0ºC. When the yellow color disappears giving a colorless and transparent solution means that the gold(I)is ready to be used in the formation of the hydrogel.

Hydrogel preparation

For a typical preparation at 5 wt % concentration, PEG-dithiol (50 mg, 14.7 µmol, 29.4 µmol theoretical thiols) was dissolved in water (708 µL). Then, 232 µL solution of Au(I) 0.1M (0.8 equivalents to theoretical sulfur) previously prepared were added to the aqueous solution of PEG-dithiol. The resulting mixture was gently shaken and allowed to stand. A transparent gel was formed after a few seconds. Then, 10 µL of a 0.07% aqueous phenol red solution was added as a pH indicator, and the pH was

[1] D. T. Hill, B. M. Sutton, A. A. Isab, T. Razi, P. J. Sadler, J. M. Trooster, G. H. M. Calis, *Inorg. Chem.*, **1983**, *22*, 2936-2942.

adjusted with NaOH 1 M (~50 µL) with vigorous shaking until the pink coloration was persistent. This resulted in a "slime" material. The acidic free-standing hydrogel was obtained by addition of a small amount of HCl 1 M to the dynamic material until yellow coloration was persistent.

To determine the effect of the amount of thiolate on the rheological properties of the dynamic hydrogel, similar procedure was carried out for the preparation of the hydrogel with 0.7, 0.6 0.5 and 0.4 equivalent of Au(I) compared to the theoretical amount of sulfur. It is also noteworthy that hydrogel prepared with 0.9 and 1.0 equivalent of Au(I) ions led to the formation of gold nanoparticles. This was attributed to the increasing viscosity of the mixture which made the formation of Au-S centers more difficult.

Characterization

Ellman test[2]

Ellman test was performed using a standard procedure reported in the literature.2 Briefly, a solution of DTNB, 2 mM and sodium acetate, 50 mM, and another solution consisting of tris(hydroxymethyl)aminomethane 1 M at pH 8 were prepared in water. Based on these two solutions, a calibration curve was carried out using N-acetyl-L-cysteine as standard. For a simple measurement, 100 µl of DTNB solution, 200 µl of Tris solution, 1680 µl of water and 20 µl of thiolated sample solution were mixed and after 5 minute incubation, optical absorbance was measured at 412 nm. The theoretical amount of thiol was calculated to be 0.588 µmol mg-1. The amount of free thiols measured with the Ellman test was 0.207 µmol mg-1. Then only 35 % of the theoretical amount of thiols are actually free thiols.

UV-visible spectroscopy. Spectra were carried out using a JASCO V-570 spectrophotometer at room temperature. 300 µL of an aqueous solution of PEG-dithiol at 10 wt.% was added to a 2 mL quartz cuvette. Spectrum of the polymer solution was initially recorded. Then, 0.1 equivalent of Au(I) ions (1.47 mL of a 0.1 solution of Au(I)) compared to the theoretical amount of free thiols were added and a spectrum was recorded after 10 min. This was repeated until adding 1.4 equivalent of gold(I).

[2] G. L. Ellman, *Arch. Biochem. Biophys*., **1959**, *82*, 70-77.

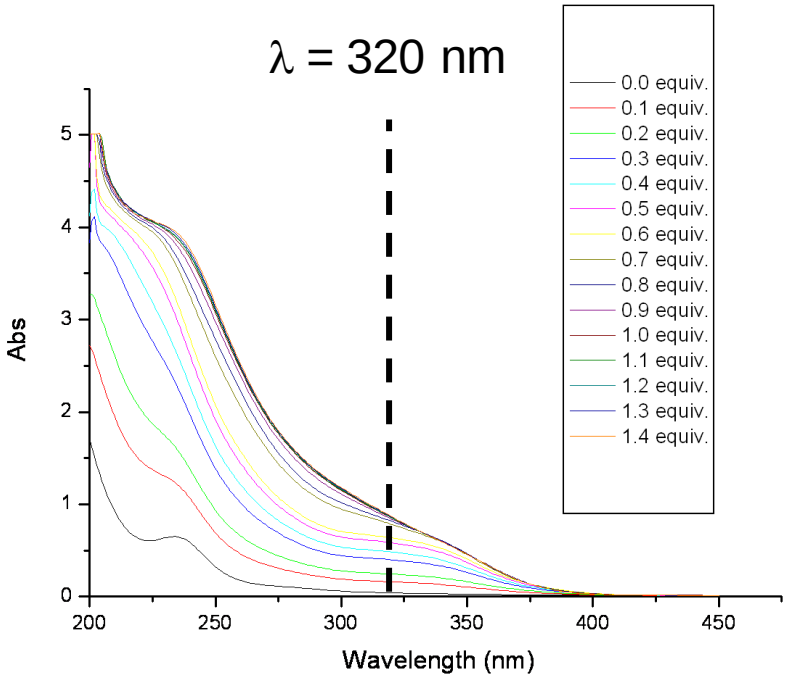

Fig. S1. Absorption curves for an aqueous solution of PEG-dithiol with added amount of Au(I). Absorbance obtained at λ = 320 nm with the amount of added Au(I) was used to prepare Figure 1 in the main manuscript.

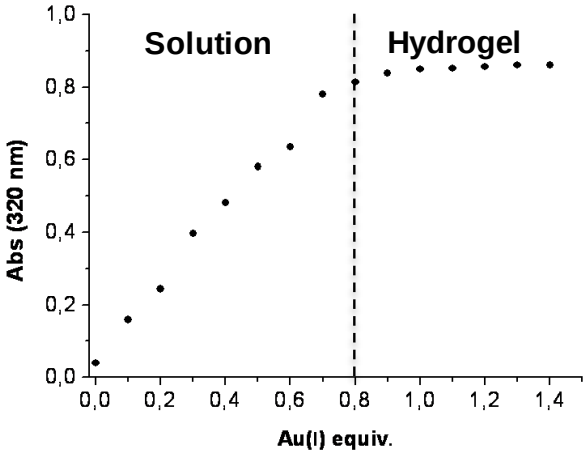

Fig. S2. Variation of the absorbance at λ = 320 nm with the equivalent number of added gold compared to the amount of free thiols for hydrogels prepared at 5 wt % PEG-dithiol.

Rheology studies. Rheological measurements were carried out using an AR2000Ex (TA Instruments) rheometer using a parallel plate (40 mm). The pH of the samples was adjusted before-hand with a Crison GLP22 pH meter. The experiments were conducted at constant temperature, i.e. 20 ºC. Storage and loss moduli (*G´* and *G"*) were recorded at constant deformation (1 %) with increasing frequency (from 0.01 to 100 Hz).

Effect of the concentration of thiolate on the thiolate/Au-S exchange. Hydrogels prepared with different equivalent of Au(I) ions and adjusted at pH 7.4 were studied by dynamic rheology. The relaxation time of each hydrogels was calculated from the frequency where G' = G'' and plotted against the inverse of the concentration of free thiolate ($[thiolate]_{free}$).

The concentration of free thiolate in each hydrogels prepared with x equivalent of Au(I) was calculated as follow:

- First the amount of Au(I) equivalent, x, allow to calculate the concentration of free sulfur in the material ($[S]_{free}$):

$$[S]_{free} = (1-x) * [S]_0 \qquad (1)$$

  where $[S]_0$ is the concentration of sulfur element calculated from the concentration of $PEG(SH)_2$ (3,400 g mol^{-1}) used to prepare the hydrogel at 5 wt.%.

- In aqueous media, sulfur can exist in two states, i.e. thiols (SH) and thiolate (S^-). Their respective concentration, [SH] and $[S^-]$ follow the equation:

$$[S]_{free} = [SH] + [S^-] \qquad (2)$$

- the pKa of thiol/thiolate was measured to be at pH 8.6 by acidic titration. Then, the acid/base couple is following the Henderson–Hasselbalch equation:

$$pH = pKa + \log_{10} ([S^-]/[SH]) \qquad (3)$$

  then the ratio between the concentration of thiolate and the concentration of thiols could be calculated from the pKa of thiols/thiolate and the pH of the hydrogel:

$$[SH] = [S^-]\ 10^{pKa-pH} \qquad (4)$$

- Combining equation (2) and (4) allow the concentration of thiolate to be calculated from:

$$[S^-] = [S]_{free} / (1 + 10^{pKa-pH}) \qquad (5)$$

- Then combining equation (5) and equation (1) allow the calculation of the concentration of thiolate as:

$$[S^-] = ((1-x)*[S]_0) / (1 + 10^{pKa-pH}) \qquad (6)$$

Where the pKa was measured at 8.6 by titration, the pH was adjusted at 7.4 for all hydrogels and $[S]_0$ was calculated as the double of the concentration of $PEG(SH)_2$ to form the hydrogel at 5 wt%.

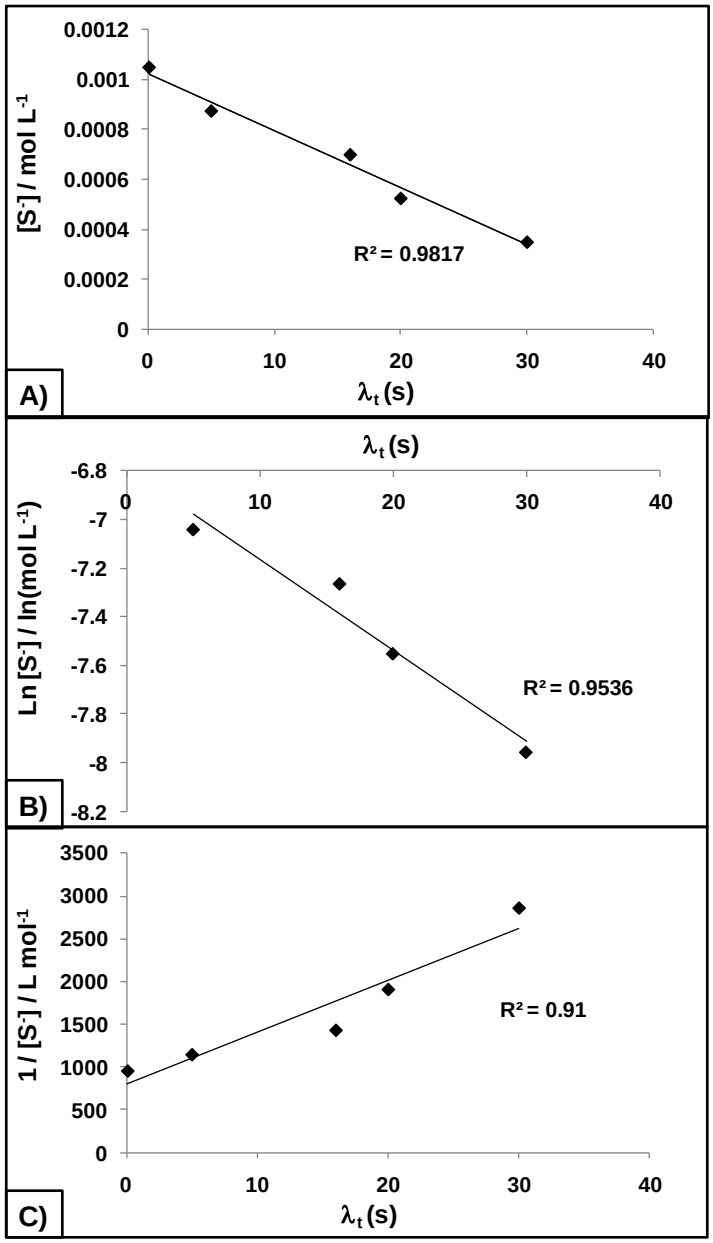

Fig S3. Variation of (A) the concentration of thiolate ($[S^-]$) *vs* relaxation time (λ_t), (B) $\ln[S^-]$ *vs* λ_t and (C) $1/[S^-]$ *vs* λ_t . The highest R^2 was obtained for graph A which indicates that thiolate/Au-S exchange follows a zero order rate reaction.

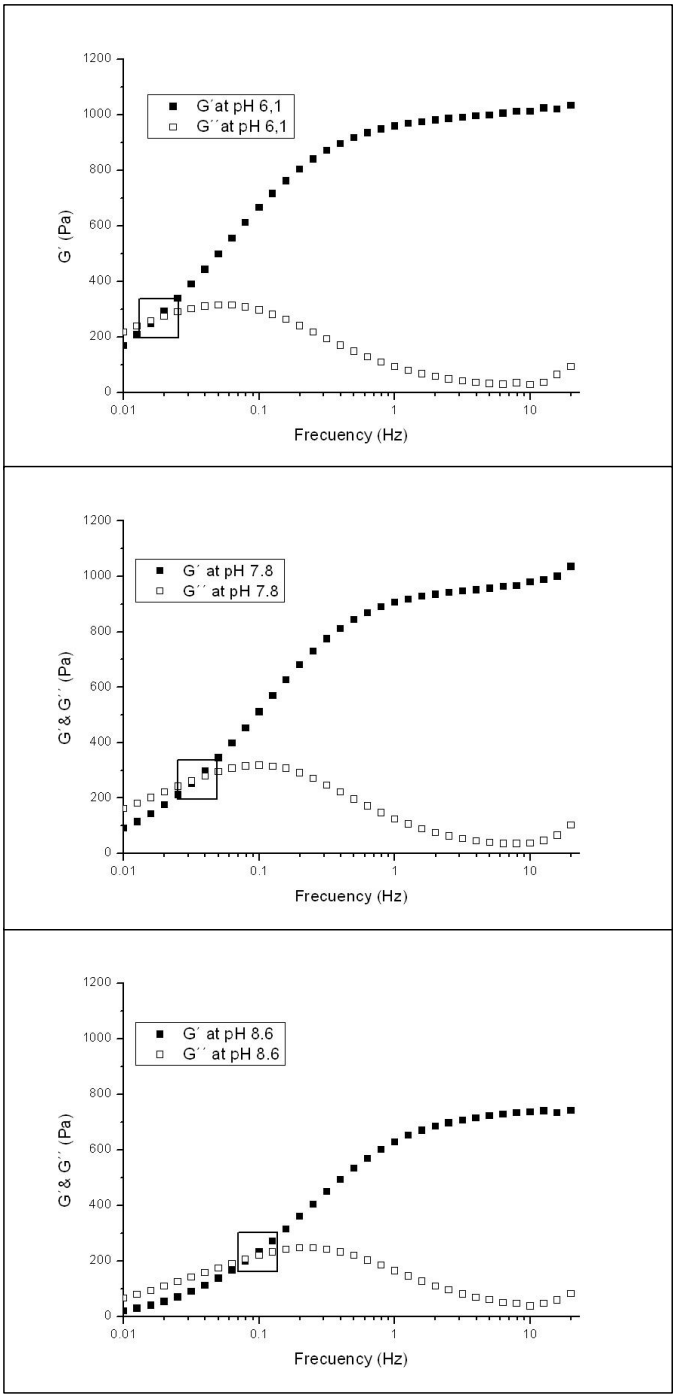

Fig. S4. Sweep frequency data of storage and loss modulus (G' and G'') for a dynamic material prepared with 5 wt % PEG-dithiol at different pH. Note the shift of the gel point (square where G' = G'') to lower frequency as the pH decreases.

In vitro studies.

Cell culture
Human Dermal Fibroblast (HDF) were obtained after informed consent from a voluntary circumcisions of healthy donors (age 24 years), following protocol approval by the relevant IRB (CEIC Gipuzkoa).

HDF cells were cultured in medium containing DMEM (Sigma), 10 % fetal bovine serum (Lonza), 1X non-essential amino acid (NEAA) (Sigma), 1 % penicillin/ streptomycin (Sigma), 2mM L glutamine (Sigma). Trypsin (0.25%)/EDTA were used to harvest the cells at 80 % confluency. Cell cultures were maintained at 37 ºC in an incubator with 95 % humidity and 5 % of CO2. The cultures were replenished with fresh medium at 37 ºC twice a week.

Determination of cell viability.

MTS assay
The effect of the hydrogel on HDF cell growth was evaluated using Cell Titer 96 ® Aqueous One Solution Cell proliferation Assay (Promega) at day 1, 3 and 7. HDF cells were seeded at a density of 3,000 cl/cm^2 in 24 well plates. The cell culture inserts (Millipore) with 200 µl of hydrogel were set in each well of HDF cultures. As a proliferation control HDF, cell culture media and Triton X-100 were used. Cells were cultured at 37 ºC of humidified incubator for 2 hours with 20µl of Cell Titer 96 ® Aqueous One Solution Reagent containing tetratzolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium)] and an electron coupling reagent, phenazine ethosulfate (PES) per 100 µl of cultured media. The absorbance per well was measured at 490 nm using a micro-plate reader (Multiscan ascent, Thermo). Cell doublings were calculated as follows: number of cell doublings = $\log_2$ (no. of cells at day 7/no. of cell seeded).

Live-Dead assay
Cell viability after hydrogel co-culture was assessed with the Live-Dead assay (Invitrogen). HDF cells were seeded at a density of 3000 cl/cm^2 in 22-mm diameter coverslips (Menzel-Glaser). Samples were processed according to the manufacturer´s recommendation. At day 7the cell culture inserts were removed from the 24 well culture plates and cells attached to the coverslips were rinsed with PBS1X (Gibco) and processed for Live-Dead assay. Cells were incubated with the ethidium-calcein mixture and incubated for 30 min at room temperature. After this treatment, the slides were prepared for microscopy on glass slides by mounting with Vectashield (Vector). A fluorescence microscope (Leica, DM 2000) and associate software were used to visualize the viability of cells.

Immunofluorescence of HDF (Ki67)
Cells attached to coverslips at day 3 were rinsed with PBS and fixed with 4% paraformaldehyde for 10 minutes, PBS-washed three times, and processed for immunofluorescence. Briefly, cells were permeabilized with 0.5% Triton X-100 for 10 minutes, washed twice with PBS, and blocked with 10% FBS (in PBS) for 20 minutes at room temperature. Primary (ki67 d (1:200); Sigma) and secondary antibody (Alexa Fluor 488, Molecular Probes) were incubated with 10% FBS (in PBS) for 1 hour at RT, with three 10

minutes PBS washes in between. Cells were washed again with PBS and nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich).

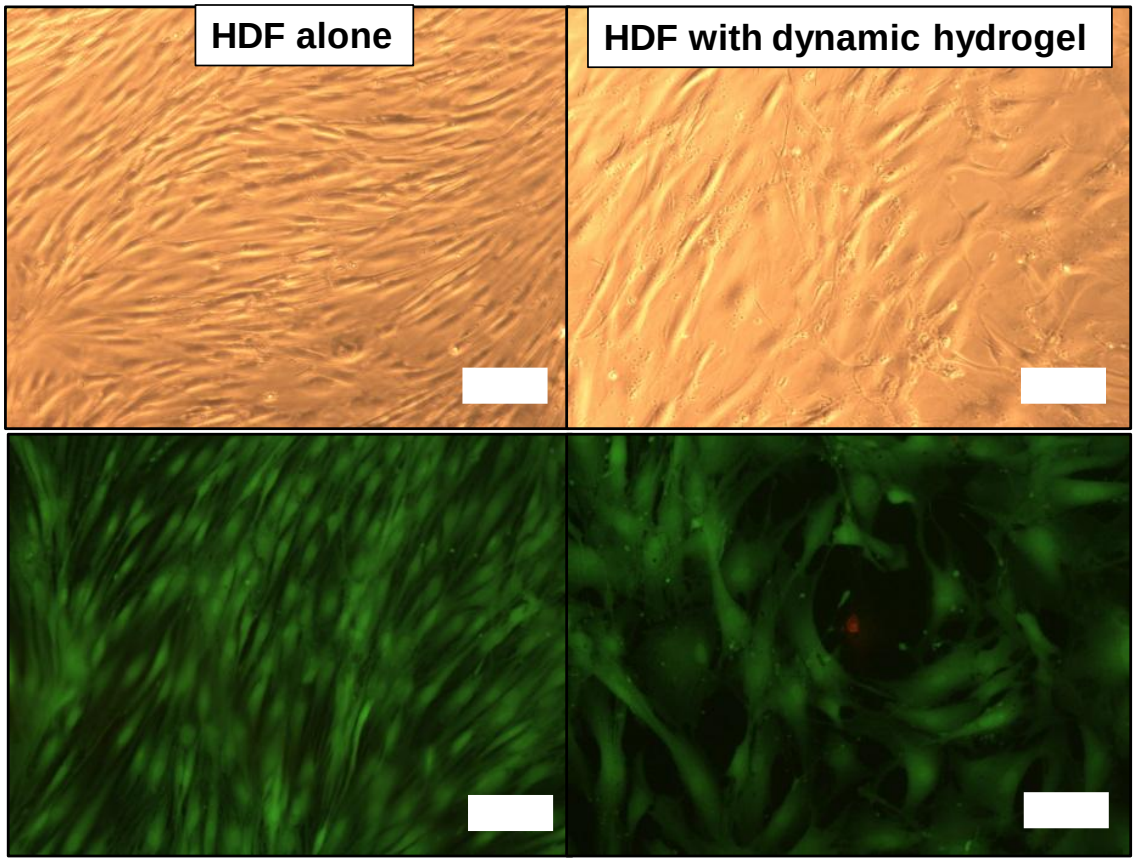

Fig. S5. (Top) Optical microscope image of HDF alone (left) and with the dynamic hydrogel (right). (Bottom) Live/Dead staining of the human dermal fibroblast alone (left) and with the dynamic hydrogel (right) at day 7. Scale bar 200 μm.

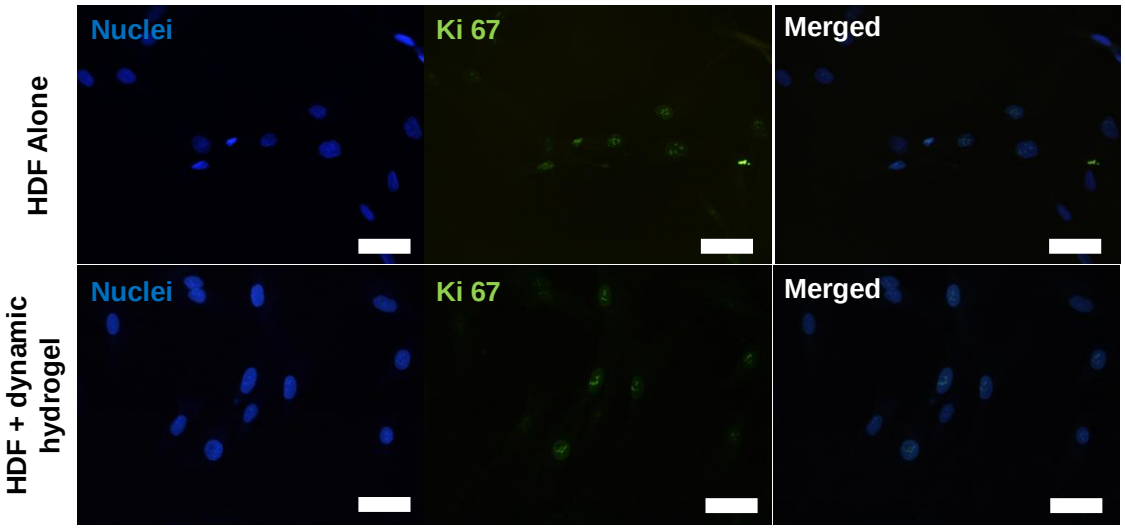

Fig. S6. (Left) Fluorescence microscope images of nuclei were counterstained with Hoechst 33258 (blue). (Middle) Fluorescence microscope images of Ki 67 staining of HDF. (Right) Both images are merged. Scale bars, 50 μm.