Electronic Supplementary Material (ESI) for Lab on a Chip.

Supporting Information Material

Study of Loctite 3525 glue as an off-the-shelf viscoelastic material for the construction of cell culture platforms in mechanobiology

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We divide the supporting information in two sections:

- details of the calculation of the mechanical properties obtained from the relaxation of the two samples
- supporting information on the material fabrication and characterization

Details of calculations of the dissipative mechanical properties.

As part of the mechanical characterization, stiffness relaxation tests were performed using the Femto Tools MTA-02 microindentation system with a 50 μm-diameter spherical tip (FT-S10000), and the temporal evolution of force under a constant deformation of 20 μm of the sample was registered. All obtained data were normalized with respect to the highest (maximal) force and the response curve was modelled using a linear viscoelastic solid, using the following equation (series of Prony):

$$F(t) = k_1\epsilon_0 + \sum_{j=1}^{n} \epsilon_0 k_j \exp(-\frac{t}{\tau_j}) \quad (1)$$

where F is the force as a function of time, k_1 is the long-range or equilibrium stiffness, k_j is the additional stiffness, $\tau_j = \frac{\eta_j}{k_j}$ is a characteristic timescale defined as the ratio between viscosity η_j and additional stiffness k_j and ϵ_0 is the initial deformation. By dividing equation (1) by the initial deformation and long-range stiffness, we obtain the following equation:

$$K(t) = 1 + \sum_{j=1}^{n} a_j \exp(-\frac{t}{\tau_j}) \quad (2)$$

where *K(t)* is the normalized stiffness and the following terms are defined by $a_j = \frac{k_j}{k_1}$. Using Wolfram Mathematica, the n^{th} order of equation (2) was selected as the best fitting when it complied with the lowest error and variance in each fitting parameter (a_j and τ_j). From equation (2) we obtained the relaxation spectrum: $h(t) = \sum_{j=1}^{n} k_j \delta(1 - \frac{t}{\tau_j})$, where the Dirac delta was approximated by: $\delta(1 - \frac{t}{\tau_j}) \approx \frac{1}{a\sqrt{\pi}} \exp(-(\frac{t - \tau_j}{a})^2)$ with $a \rightarrow a_j$ instead of $a \rightarrow 0$.

Once the fitting was obtained, the effective characteristic timescale was chosen to model the material as a standard linear solid (SLS), modifying equation (2) into the following:

$$k(t) = k_l + k_a \exp\left(-\frac{t}{\tau_a}\right) \quad (3)$$

reminding that this equation (3) is normalized by k_t+k_a.

Using the Boussinesq-Green's function approach [1,2], we obtain that the substrate stiffness k is proportional to the elastic modulus E: $k(t) \approx 2RE(t)$ using a characteristic length scale of $2R \approx 1\mu m$ (considering a typical focal adhesion is in this order of magnitude) and assuming a Poisson's ratio of 0.5. And by substituting $\cosh(x) + \sinh(x) = \exp(x)$ in equation (3), we finally obtain:

$$E'(t) = E_l + E_a \cosh\left(-\frac{t}{\tau_a}\right) \quad (4a)$$

$$E''(t) = E_a \sinh\left(-\frac{t}{\tau_a}\right) \quad (4b)$$

To calculate *E'* and *E''*, we used a characteristic binding timescale of 1s for a focal adhesion [2,3]; the a_j, τ_j values were then obtained from the fitting of our normalized experimental curves; $E_l = k_l/1\mu m$ and $E_a = k_a/1\mu m$ enable the calculation of the dynamic module of the material taking into account a focal adhesion binding timescale and characteristic length. It is important to remark here that the Laplace transform of equations (4a) and (4b) giving $E'(\omega)$ and $E''(\omega)$ is consistent to what is reported in the literature [2].

Additional figures and supporting information on fabrication and characterization

We have measured the UV-visible absorption spectra of Loctite 3525 after 2s and 40s of UV exposure in the NIL system. It is visible that absorption decreases when cross-linking increases. We also show the autofluorescence emission when excited at 365 nm.

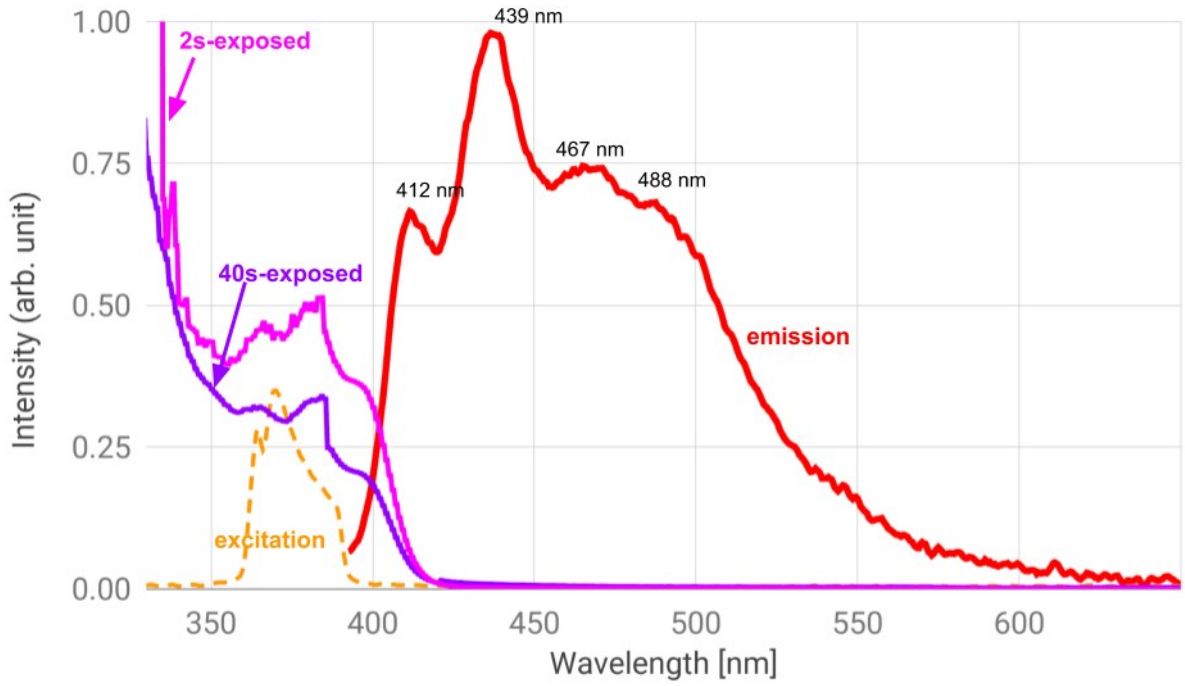

Figure S1. UV-Vis absorption and auto-fluorescence emission spectra of 200µm thick layers of exposed Loctite 3525. 365 nm excitation source used for auto-fluorescence measurement is shown.

We have also measured Fourier Transform Infrared (FTIR) transmission to compare the composition of the surface of the resin after 2s and 40s cure and discard that the response of the cell was caused by the presence of certain adhesion promoters such as amine groups for instance. Measurements were performed using Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), using a Perkin Elmer Spectrum Two system on samples treated for cell culture and no significant differences were observed. We looked at regions characteristic of amine group bands: N–H stretch (from primary and secondary amines), N–H bend (primary amines only), N–H wag (primary and secondary amines only) and C–N stretch (aromatic and aliphatic amines). It was apparent from our experiments that poly-L-lysine was required for the cells to attach and Figure S2 shows that there is no difference between the Loctite 3525 cured for 2s and 40s in the NIL system, and no amine bands were detected.

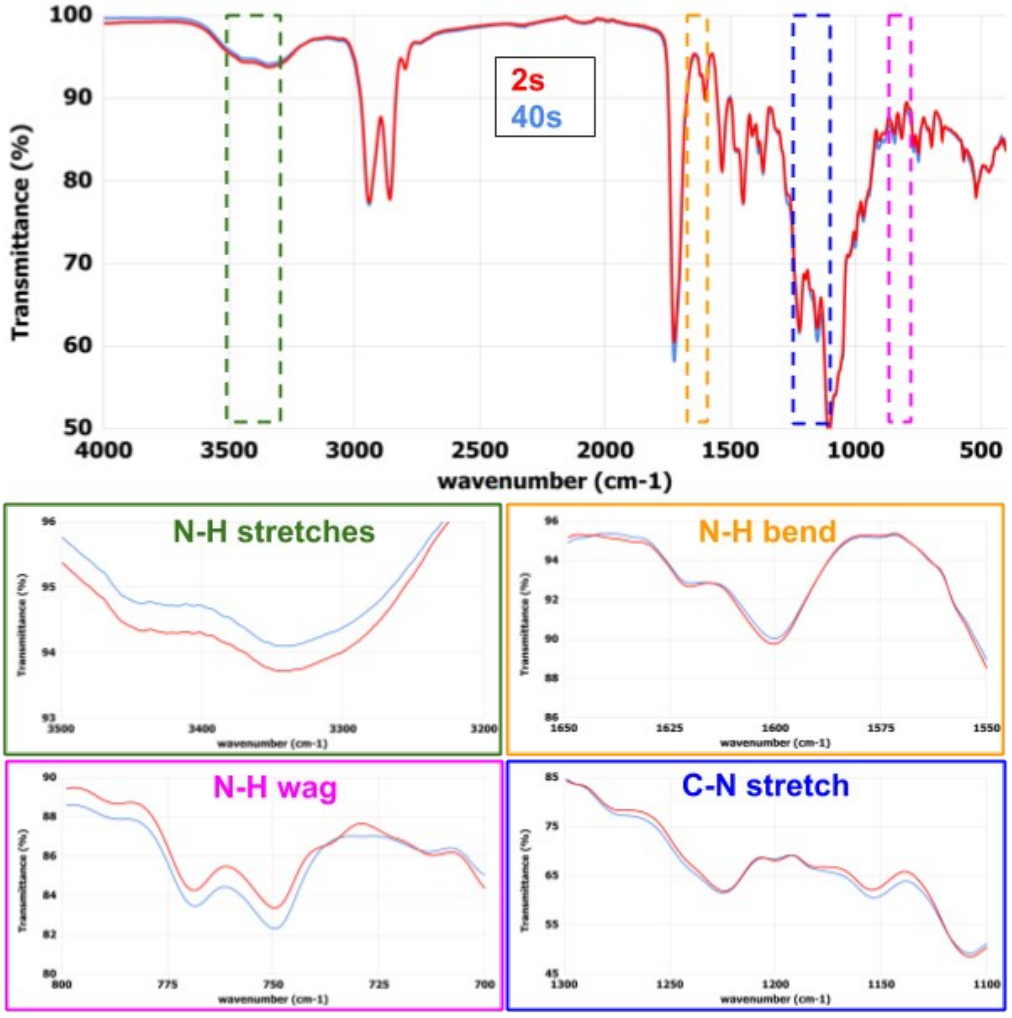

Figure S2. FTIR spectra of 2s and 40s cured Loctite 3525 with emphasis on amines.

We also assessed cross-linking kinetics using FTIR spectra [4]. Indeed, the transmission of the band centered at 816 cm^{-1} in relation to the 845 cm^{-1} centered band is monitored to assess polymerization of acrylate resins. Apart from this region and the -OH bands, no difference was found between the spectra. As can be seen in Figure S3, $\frac{T_{816}^{2s}}{T_{845}^{2s}} \leqslant 1$ while $\frac{T_{816}^{40s}}{T_{845}^{40s}} \succ 1$, proving that the resin is not fully cross-linked after only 2s of exposure.

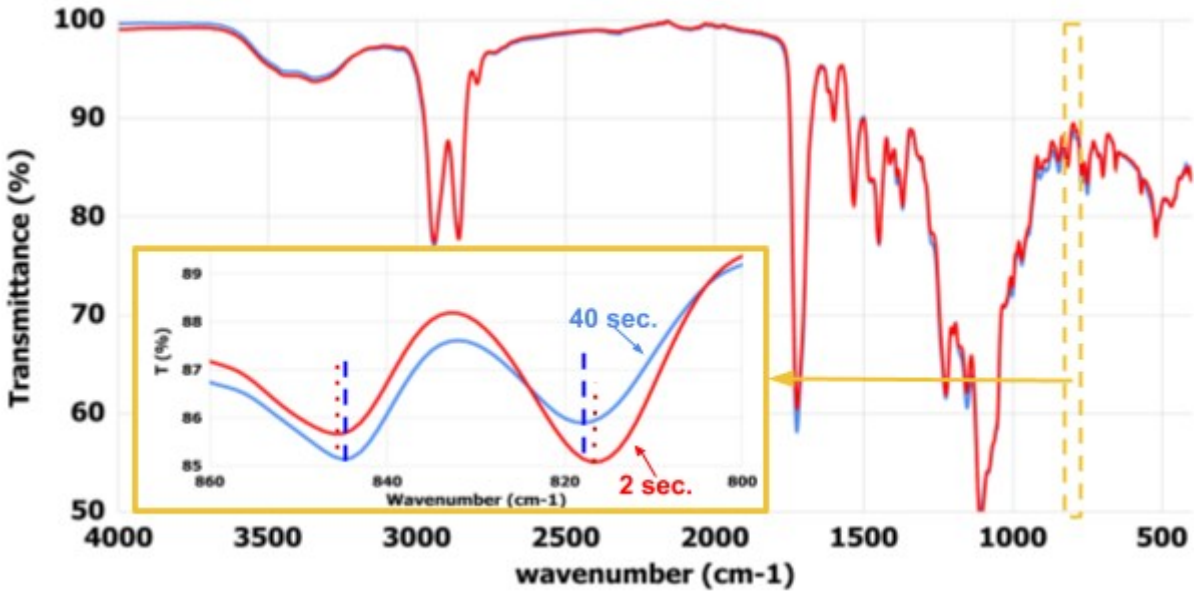

Figure S3. Detail of FTIR spectra of 2s and 40s-exposed Loctite 3525 layers.

It was possible to integrate hydrogels inside PDMS microfluidic chip thanks to Loctite 3525, as depicted in Figure 6 of the article. The substrates hosting the Loctite layers were oxidized with 0.1 M of NaOH since it promoted the adhesion of Loctite when those were subsequently submerged in culture medium for prolonged times. Then a droplet (0.3 gram) of Loctite 3525 was placed on a coverslip and covered with a thin transparent acetate sheet to expand the viscous droplet and ensure flatness of the cured resin layer after UV dose. We tried different UV sources obtaining good results, so the NIL system is not necessary and could even result counterproductive for such applications.

Apart from PAAm hydrogels crosslinked chemically, we have managed to use poly(ethylene glycol) diacrylate (PEG-DA) hydrogels over Loctite and PEG-DA attached strongly to the Loctite-covered coverslips. PEG-DA and PAAm crosslinked using UV light and Irgacure 2959 as photoinitiator were cured as follows:

- *PAAm-UV*: 100µl of acrylamide were mixed with 66µl of bisacrylamide in 284µl of water and 50µl of Irgacure 2959. This resulted in a 20 kPa gel.
- *PEG-DA-UV*: here we mixed 400µl of PEG-DA in 600µl of water and 100µl of Irgacure 2959

Figure S4 below shows the detailed procedure to construct a PDMS chip with hydrogel integrated inside the channel:

1. First a PMMA micromold was fabricated with the following dimensions 1mm (W) x 1cm (L) x 150µm (D) using laser ablation. Using replica-molding, we obtained the negative pattern inside a PDMS slab (Sylgard 184, in a 10:1 w/w proportion of prepolymer/curing agent). After silanizing this slab with dichloromethylsilane vapor (1h with 1ml in a closed chamber), we used the replica-molding technique once again to obtain the laser ablated pattern inside another PDMS slab. that will serve as one side of the final microchannel.

2. In order to deposit Loctite 3525 locally, we used a perforated adhesive tape leaving an open window above the channel (Figure S4.A), in which we injected a small volume of Loctite 3525 (Figure S4.B), Excess was removed using doctor blading (Figure S4.C) before the tape was carefully removed (Figure S4.D) and a second time after removal to fill the channel with Loctite and avoid excess. In Figure S4.E, we used Loctite mixed with natural blue colorant in order to visualize the material. This colorant did not impede photocuring.

3. A 100µl droplet of hydrogel was then deposited on the laser etched PMMA micromold that contained the exact same microchannel of the PDMS slab where the Loctite 3525 resin had been cured (Figure S4.F). Details of the microstructuring of hydrogels using PMMA molds will be detailed in another report. The Loctite-containing PDMS slab was then carefully aligned with the PMMA mold with the hydrogel droplet and cured either with TEMED or UV-light. The PMMA mold was then finally removed, leaving the hydrogel on top of Loctite micropattern (Figure S4.G). As hydrogels do not adhere on PMMA nor crosslink on PDMS due to the oxygen inhibition layer, the hydrogel follows the Loctite micropattern strictly.

4. The last step consisted in sealing the PDMS channel where a similar channel was placed onto the PDMS-Loctite-hydrogel layer (Figure S4.H).

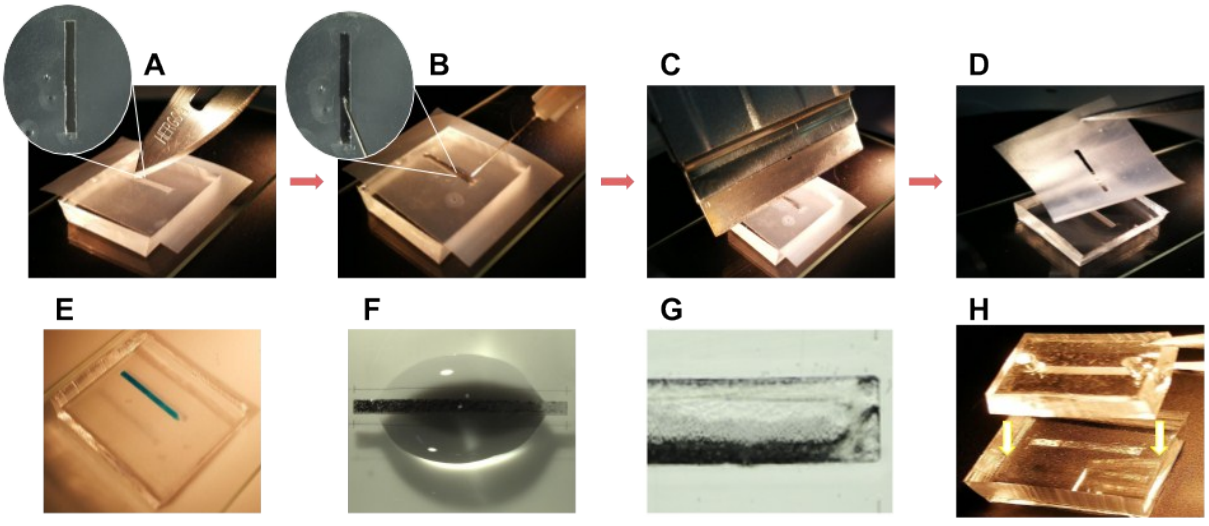

Figure S4. Details of the simple construction of PDMS microchannel integrating hydrogel thanks to Loctite 3525. A PDMS slab containing a microchannel was coated with an adhesive tape to open a window (A) where Loctite is injected locally (B). Excess is removed by doctor blading (C) both before and after removal of the tape (D). Loctite may be colored using natural colorant in order to visualize it after crosslinking (E). Then a 100µl droplet of the prepared

hydrogel is placed on the microstructured PMMA mold that was used to construct the PDMS slab used to host Loctite (F) and is crosslinked by placing the PDMS slab on top of it. When PMMA is removed, the hydrogel remains only where Loctite is present (G). Finally, the PDMS microchip may be sealed using a conventional technique (H).

In order to compare the adhesion of hydrogels on Loctite with other substrates, we have performed some peel-tests using a homemade system measuring traction forces between two parallel plates. The system was automated to press the two plates upon the gels and the substrate and subsequently control the separation between the two plates in a stepwise fashion elongating the gels and measuring the force. Although the test is not following the exact conditions of uniaxial tensile test, it was an excellent assay to analyze the adhesion of the gels until detachment and comparison between Loctite and typical treated glass substrates. Figure S5 shows examples of representative results of adhesion obtained for PAAm gels on Loctite coating glass (blue) and on APTES-glutaraldehyde treated glass coverslips (red). Hydrogels were deposited on glass without treatment as a non-adhesion control (orange). It is clear that Loctite is an excellent substrate to host hydrogels, better even than treated glass coverslips that we usually use.

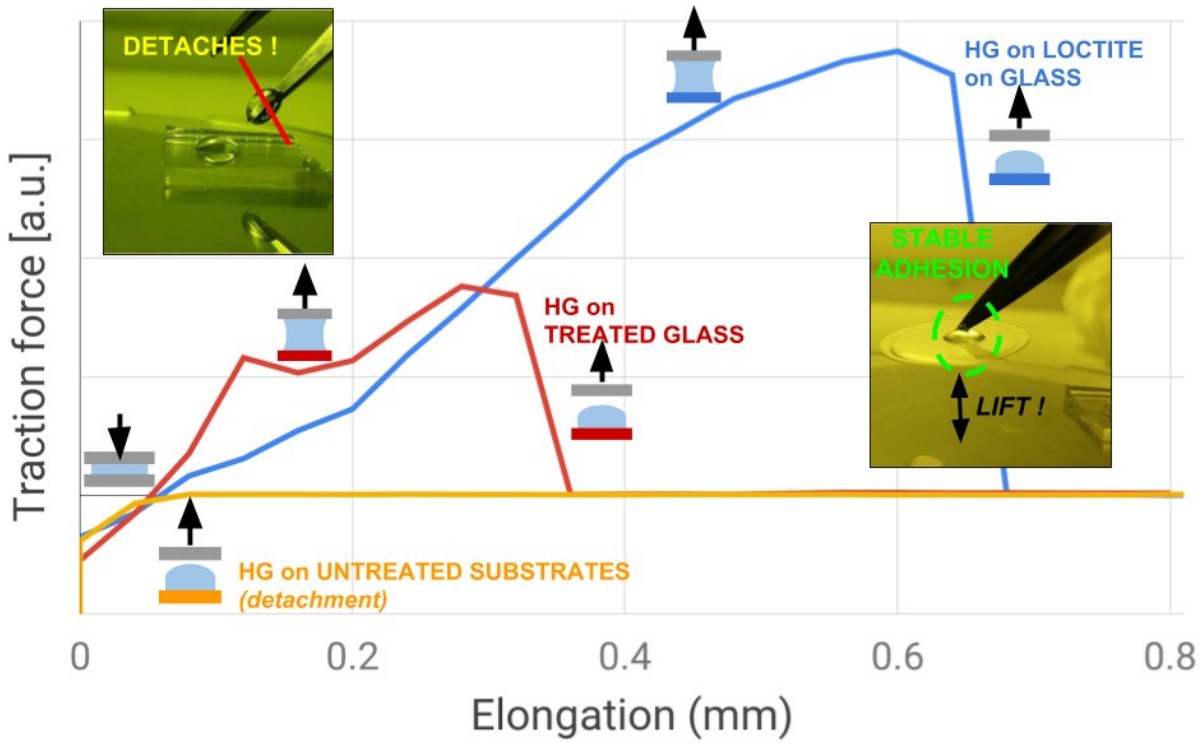

Figure S5. Qualitative traction force tests of PAAm gels on different adhesion substrates. Inlets are representative of gels adhesion, using tweezers.

Following the procedure detailed in Figure S3, it was possible to integrate hydrogel as a diffusion barrier for co-culture inside a PDMS channel (Figure S6). In this case, a PDMS spacer was used to separate two similar PDMS slabs containing Loctite and benefit from the hydrogel crosslinking inhibition of PDMS. Indeed, here the prepared hydrogel was injected inside the channel shown in Figure S6.A. After crosslinking and rinsing inside PDMS, hydrogel attached only to Loctite, leaving two parallel channels that could be used to construct a co-culture chip (Figure S6.B). Successful preliminary results were obtained using HepG2 and 3T3 cells separated by the hydrogel diffusion barrier which thickness can be easily controlled by the control of Loctite micropatterns widths. More work is currently underway for this particular application.

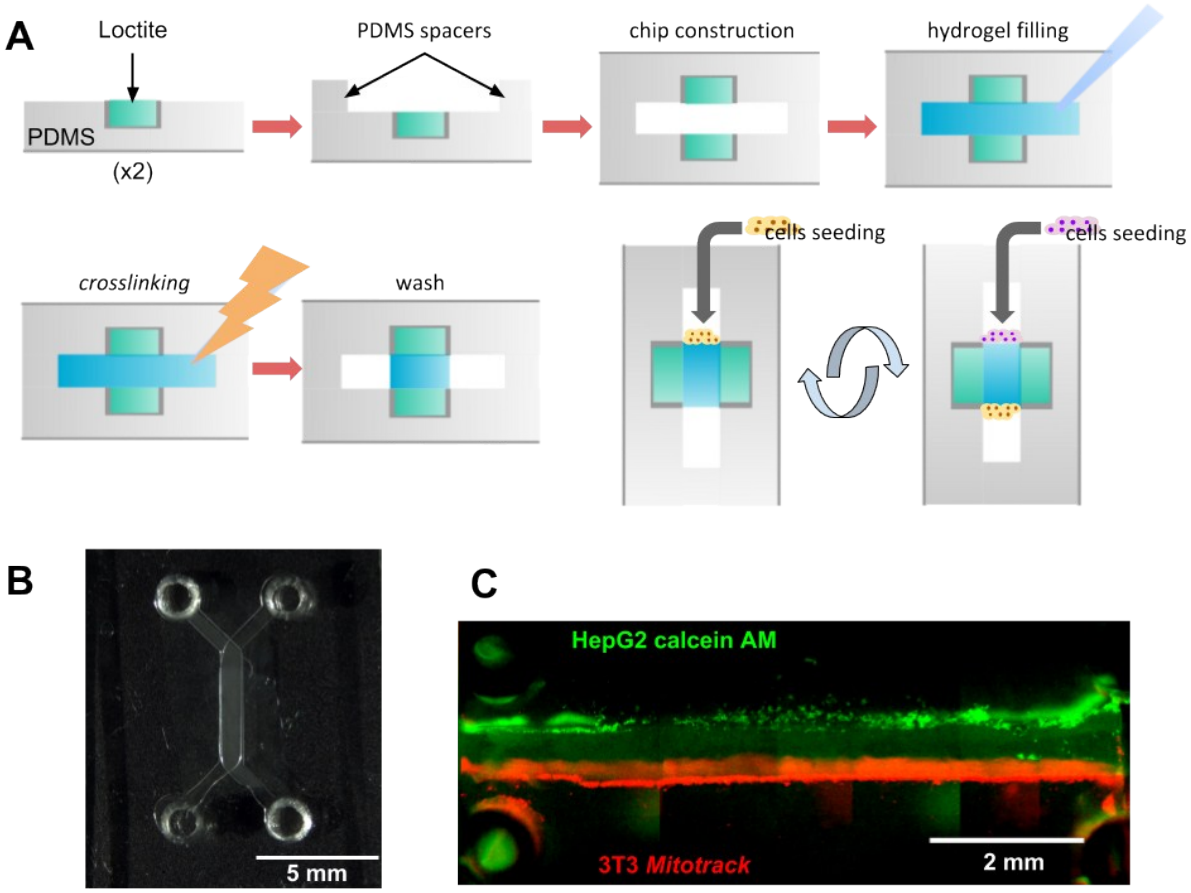

Figure S6. Construction of hydrogel diffusion barrier inside a PDMS channel (A). Proof of concept of co-culture using HepG2 and 3T3 cells (B).

When hydrogels were attached to Loctite 3525, it was very important to follow a critical step

to avoid wrinkling of the gels surface, as depicted in Figure S6. According to the literature [5] the wrinkles are caused by an expansion difference between the substrate and the gels when hydrated. This is an issue as cells followed these patterns on the surface and the size of the wrinkles led to great light scattering impeding microscopy. In order to avoid this effect, Loctite had to be previously hydrated in water or PBS, at least 2 hours before hydrogels were placed. We indeed believe Loctite swelled when hydrated, and that the expansion caused by hydration beforehand was sufficient to avoid more swelling with hydrogel moisture.

We also found that the exposure time to cure Loctite affected the wavelength of these wrinkles, similarly to what had been reported [5]. Interestingly, the wavelength obtained in hydrogels on less-cured Loctite was measured to be between 1.5 and 2 times smaller than those found on fully cured Loctite.

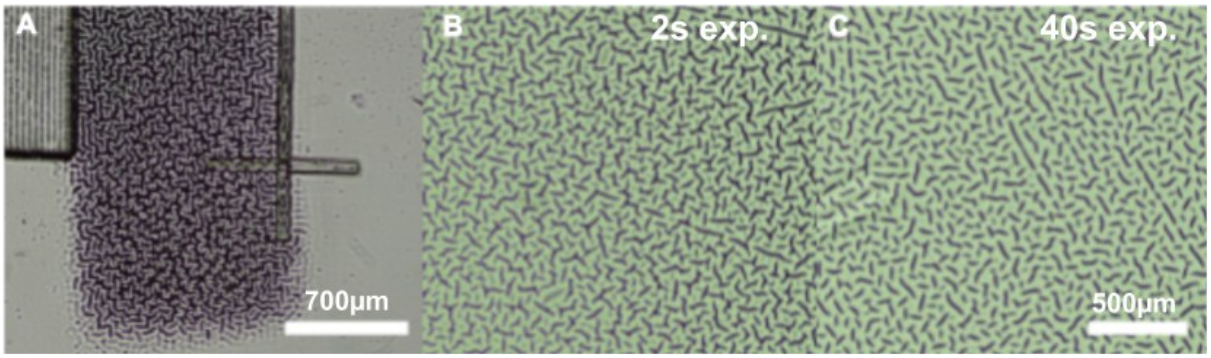

Figure S7. Micrographs of hydrogels wrinkling on Loctite substrates: on a micropattern (A), after 2s of exposure (B) and after 40s of exposure (C).

To discard apoptosis processes in 3T3-L1 fibroblasts cultured on Loctite UV cured resins, we used Apoptag® apoptosis detection kit based in TUNEL assay to evaluate cell viability. Some UV cross-linked resins could be partially cured during polymerization process and some free components could get into the medium intoxicating directly the cells attached to the surface. As we expected, neither 2s nor 40s cured Loctite presented apoptotic fibroblast even during 48h of culture. This finding places Loctite 3525 as a compatible UV sensitive resin for cell culture and mechanobiology assays as well as a new material suitable for microfabrication in cell culture platforms. Doxorubicin was used as a control for apoptosis induction as it was previously described for 3T3-L1 fibroblasts [6].

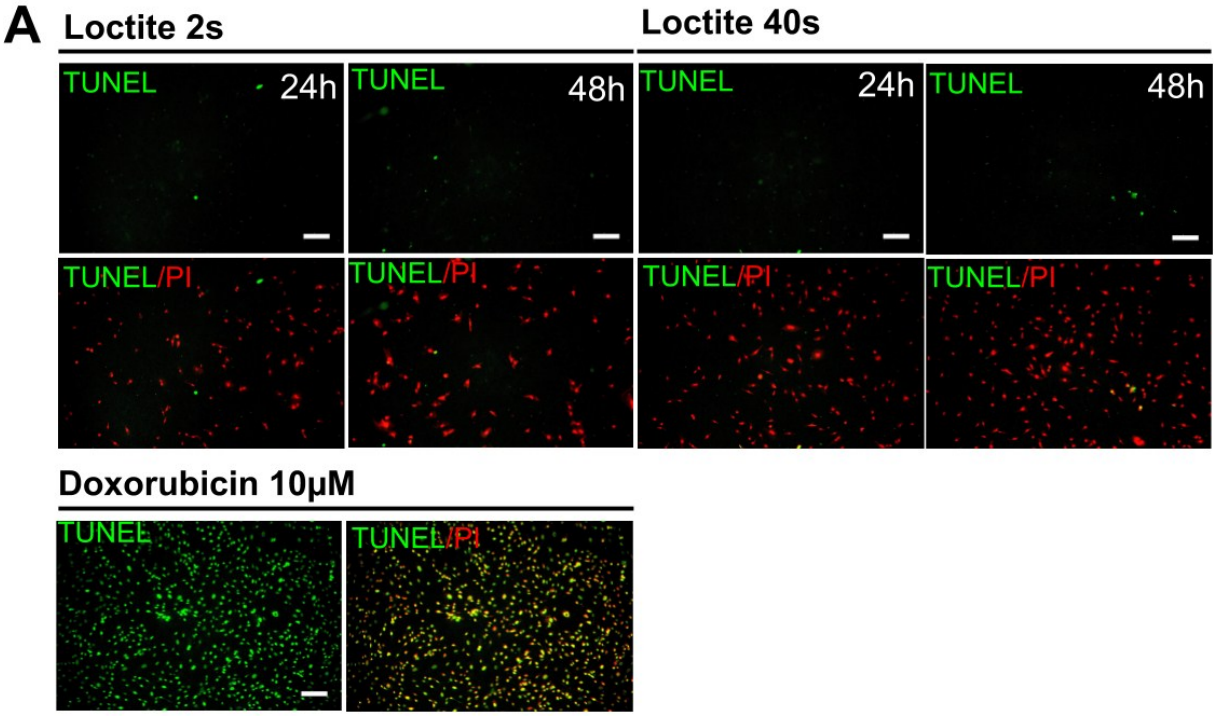

Figure S8. Apoptosis detection assay for 3T3-L1 mouse embryonic fibroblasts cultured on differentially cross-linked Loctite. (A) Loctite was exposed with 365 nm UV light for 2 and 40 seconds in a NIL system and 3T3-L1 Fibroblast were cultured for 48 hours. Apoptotic cells were detected by using Apoptag® fluorescein *in situ* apoptosis detection kit based in terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay. Doxorubicin was used as a control of apoptosis induction in 3T3-L1 cultured fibroblasts. Nuclei were detected by propidium iodide (PI) staining. Apoptosis detection assay is a representative of 2 independent experiments. Scale bar = 200µm.

Additionally to YAP/TAZ nuclear translocation analysis in 3T3-L1 mouse embryonic fibroblasts cultured on 2s and 40s UV exposed Loctite, we decided to analyze ß-catenin protein under the same conditions. We observed a very clear ß-catenin signal in cell to cell contacts (cell junctions) in fibroblasts cultured over glass coverslips covered with Poly-L-lysine. However, not only ß-catenin signal was reduced (shown in the heat map) but also the localization of ß-catenin in cell junctions was partially and totally lost in 40s UV and 2s UV exposed Loctite, respectively. This is relevant because besides YAP/TAZ mechanosensitive response, we observed another mechanotransduction signal that responded to the mechanical differences that 2s and 40s UV cured Loctite clearly presented. We also demonstrated that YAP/TAZ signaling was also modulated in 3T3-L1 fibroblasts cultured in PAAm hydrogels polymerized on 40s UV exposed Loctite-covered glass coverslips. First, we cultured fibroblasts on 1 kPa and 20 kPa PAAm hydrogels and detected actin filaments to demonstrate the mechanical response of fibroblasts under these conditions. 3T3 Fibroblasts spreaded significantly on 20 kPa hydrogels compared with fibroblasts cultured on 1 kPa hydrogels. As previously reported, YAP/TAZ proteins translocated to cytoplasm in the soft substrate where the cell confinement of fibroblasts was evident. YAP/TAZ protein translocated to nucleus in 3T3-L1 fibroblasts cultured on stiff PAAm hydrogels (20 kPa).

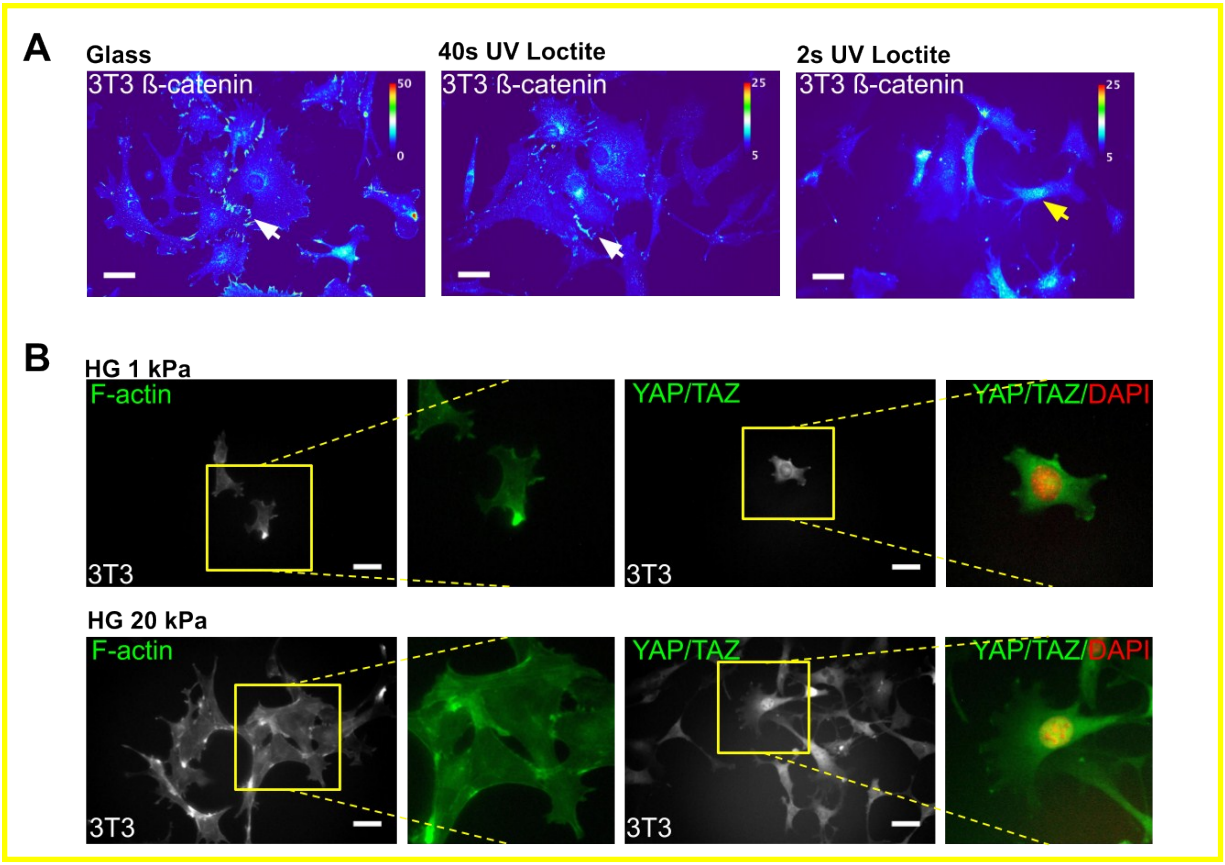

Figure S9. Mechanoresponse of 3T3-L1 Fibroblast on Loctite and PAAm hydrogels (HG). (A) ß-catenin localization in fibroblasts cultured on differential UV exposed loctite. Heat map of ß-catenin signal density calibrated from 0 to 50 for glass and from 5 to 25 for 2s and 40s UV exposed Loctite. White arrows point ß-catenin signal present in cell to cell contact; yellow arrow points ß-catenin signal located in the cytoplasm. Scale bar = 50 µm. (B) Culture of 3T3 fibroblasts on PAAm HG. F-actin was detected by Alexa Fluor 488 phalloidin (green) and YAP/TAZ proteins were detected by immunofluorescence (green); nucleus was detected by DAPI (red). Scale bar = 50 µm.

References of Supporting Information

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