Abstract
Epithelial and other cells in soft tissues adhere to a microenvironment whose stiffness typically falls in kilopascal range. Flexible substrates such as polyacrylamide and silicone gels have proven to be excellent biomimetic substrates for cell culture. To characterize the Young’s moduli of isotropic linear elastic substrates, we present here a simple method that only employs a widefield fluorescence microscope for the actual stiffness testing. Use of suitable indentors and methods to restrict fluorescent marker beads to the substrate top surface enable this method.

Background
Several methods have been used to measure the stiffness of flexible substrates, including atomic force microscopy, macroscopic deformation of whole samples upon stretching, rheology and indentation using spheres and spherically tipped microindentors. While each technique has its own advantages and disadvantages, indentation with a sphere is an especially simple yet fairly accurate method that only requires access to a widefield fluorescence microscope. Recently, confocal microscopy has also been used for an elegant characterization of the indenter method [1]. Here, we show that a widefield microscope may be sufficient, provided a suitable protocol is used to confine fluorescent beads to the top surface or to a thin slice in the top region of the substrate.

Methods
Polyacrylamide (PAA) gels were prepared on a coverslip by polymerizing acrylamide and bis-acrylamide solutions in water with polymerizing chemical agents for 45 minutes. A thin top layer of PAA solution with fluorescent beads was then polymerized on the previously formed polymerized gel. Soft silicone was prepared in a petri dish by mixing the base and curing agent at a 1:1 ratio followed by curing at 70 degrees C for half an hour. Fluorescent beads of 0.44 micron diameter were coupled to the silicone top surface. Various parameters of interest are indicated. (B) This panel shows an image of a 1 mm indentor (on a soft silicone sample) obtained via phase imaging. The scale bar indicates 250 μm.

Results
Fluorescent image of the microbeads in the top surface of the silicone as taken with a widefield microscope is shown in Fig. 4. Z-stack of the regions under the indentor and away from the indentor are shown as well. For the 1:1 soft silicone, the Young's modulus we obtained was 7.2 ± 2.4 kPa. We also showed that decreasing the silicone mix ratio yielded higher stiffness (37.6 ± 3.9 kPa for 4:7 and 64.1 ± 6.9 kPa for 1:2). To calculate the Young's Modulus, we used a modified [2] Hertz equation:

\[ E = \frac{c(1-v^2)F}{4R\delta^{1.5}} \]

Where:
- \( c \) = a correction factor that modifies the Hertz model expression that follows it;
- \( v \) = Poisson’s ratio of the silicone gel (taken as 0.5 as for incompressible materials);
- \( F \) = the indentation force;
- \( R \) = the indentor radius; and
- \( \delta \) = the indentation depth

Figure 3: Schematic illustration of sphere indentation on the soft silicone surface. (A) This schematic depiction shows a spherical indentor on the surface of a soft silicone sample. Various parameters of interest are indicated. (B) This panel shows an image of a 1 mm indentor (on a soft silicone sample) obtained via phase imaging. The scale bar indicates 250 μm.

Figure 4: Bead image acquisition and determination of the in-focus image. (A) This fluorescence image shows microbeads on the top surface of the soft silicone sample and the desired x-y location of its frame relative to the indentor (dotted line). The scale bar indicates 150 μm. Panels B and C show z-stack fluorescence images of regions on the soft silicone sample (B) under the indentor and (C) away from the indentor (boxed regions in the top image). The indicators z1 and z2 correspond to the z-values at which the region under the indentor and the region away from the indentor are in focus, respectively. The scale bars indicate 20 μm. The monochrome images shown are those obtained in the red channel since nominally pink microbeads were used whose excitation and emission profiles fit the red channel. (D) This panel shows an intensity line scan across a micro-bead (shown in the inset image with a yellow line across it) as the focus is varied in z-increments of 0.5 μm. The focus (z-value) corresponding to the in-focus image can be objectively chosen based on the z-value corresponding to the line scan with the highest maximum intensity. The scale bar in the inset indicates 20 μm. Repeat of such scans for several nearby beads can be used to obtain an improved estimate of the in-focus z-value.

Conclusions
We have presented a relatively simple method to couple fluorescent beads to the soft silicone top surface (or a PAA gel top layer) and measure its Young’s modulus using an indentor, from data taken with a widefield fluorescence microscope. Common availability of this equipment makes this method widely accessible.

References