Overview comment: The manuscript titled “Non-invasive nanometer resolution assessment of cell-soft-hydrogel system mechanical properties by scanning ion-conductance microscopy” by Tikhonova, T.N.; et al. is a scientific work where the authors combine scanning ion conductance microscopy (SICM) and atomic force microscopy (AFM) to extract the elastic modulus of carcinogenic MCF-7 breast cancer cells seeded on substrates with different stiffness. This study could be interesting to optimize and standardize protocols concerning how the substract can impacts on the Young’s modulus of the examined cellular samples. The manuscript is generally well-written and this is a topic of growing interest.

Response: We thank the reviewer for the positive assessment of the work and appreciate the time and effort invested.

All the corrections that were added into the manuscript according to the Reviewer suggesting were highlighted with yellow.

Comments 1: “(…) ‘soft substrate (…) (lines 33-34). The authors need to fix this grammar issue.

Response 1: The term "soft substrate effect" was taken from the composite model proposed by Rheinlaender et al. [Rheinlaender 2020], which was developed to account for the additional deformation of the substrate that may occur in a cell-soft substrate system, where the substrate is softer than the cell. This model is described in Discussion section and used for AFM data processing.

- Rheinlaender, J.; Dimitracopoulos, A.; Wallmeyer, B.; Kronenberg, N.M.; Chalut, K.J.; Gather, M.C.; Betz, T.; Charras, G.; Franze, K. Cortical cell stiffness is independent of substrate mechanics. *Nat. Mater*. **2020**, *19*, 1019-1025.

Comments 2: Keywords. The authors should consider to add the term “MCF-7 breast cancer cells” in the keyword list.

Response 2: The term “MCF-7 breast cancer cells” was added to the keyword list.

Comments 3: “Hydrogels can be broadly categorized into synthetic and natural materials. Synthetic hydrogels, engineered from polymers (…) crosslinking density” (lines 52-54). Could the authors provide some illustrative examples?

Response 3: The illustrative examples of synthetic hydrogels were added into the sentence: “Synthetic hydrogels, engineered from polymers such as polyethene glycol and polyamides offer the advantage of tunability, where their mechanical properties can be precisely controlled by altering the crosslinking density [15].”

Comments 4: Then, during the Introduction section it is also necessary to discuss about the existence of other alternative techniques to ascertain the mechanical properties of the examined sample. In this framework, optical tweezers [1] and magnetic resonance elastography [2] need to be mentioned highlighting how the combination of AFM and SICM offer advantages compared to the above described tools.

[1] Magazzù, A.; et al. Investigation of Soft Matter Nanomechanics by Atomic Force Microscopy and Optical Tweezers: A Comprehensive Review. *Nanomaterials* **2023**, *13*, 963. https://doi.org/10.3390/nano13060963

[2] Kim, S.H.; et al. Magnetic Resonance Elastography for the Detection and Classification of Prostate Cancer. *Cancers* **2024**, *16*, 3494. https://doi.org/10.3390/cancers16203494

Response 4:

In recent years, the exploration of mechanical properties of materials at the nanoscale has garnered significant attention, leading to the development and application of various techniques in addition to the traditional, well-recognized AFM method. Among these, optical tweezers, as reviewed by Magazzù et al. [Magazzu], utilize highly focused laser beams to manipulate and measure the mechanical properties of soft matter at the nanoscale. For instance, optical tweezers have been used to study changes in the characteristic elasticity of cells associated with various human diseases [Dao 2003, Agrawal 2016]. This technique is particularly advantageous for its non-invasive nature and high precision; however, it is effective only for manipulating very small objects, such as microparticles and molecules, which limits its application to larger samples. Additionally, the method's effectiveness decreases when working with thicker or opaque samples. Methods are also being developed that can be used directly in clinical settings to determine tissue stiffness in patients with diseases such as prostate cancer [Kim 2024]. One such method is magnetic resonance elastography. Although this technique offers a non-invasive imaging method that measures the mechanical properties of tissues, making it valuable for medical applications, it has lower spatial resolution compared to methods like optical tweezers or AFM, which may limit its application for studying small structures.

- Magazzù, A.; et al. Investigation of Soft Matter Nanomechanics by Atomic Force Microscopy and Optical Tweezers: A Comprehensive Review. *Nanomaterials* **2023**, *13*, 963. https://doi.org/10.3390/nano13060963

-Dao, M.; Lim, C.T.; Suresh, S. Mechanics of the human red blood cell deformed by optical tweezers. *J. Mech. Phys. Solids* **2003**, *51*, 2259–2280.

-Agrawal, R.; Smart, T.; Nobre-Cardoso, J.; Richards, C.; Bhatnagar, R.; Tufail, A.; Shima, D.; Jones, P.H.; Pavesio, C. Assessment of red blood cell deformability in type 2 diabetes mellitus and diabetic retinopathy by dual optical tweezers stretching technique. *Sci. Rep.* **2016**, *6*, 15873.

- Kim, S.H.; et al. Magnetic Resonance Elastography for the Detection and Classification of Prostate Cancer. *Cancers* **2024**, *16*, 3494. https://doi.org/10.3390/cancers16203494

Comments 5: Finally, could the authors provide quantitative data insights according to the worldwide global incidence burdens of breast cancer malignancies? This will significantly aid the potential readers to better understand the significance of this research.

Response 5:

In this study, MCF-7 breast cancer cells were chosen as a convenient and well-researched model for investigating the mechanical properties of the cell-soft hydrogel system using a novel alternative method, SICM. For instance, in study [39], the morphology of MCF-7 cell membranes

was examined in relation to substrate stiffness, specifically changes in the number of microvesicles, microvilli, and the structure of filopodia. Gil-Redondo et al. demonstrated changes in the shape of these cells depending on the substrate stiffness [53]. It was hypothesized that if this convenient model could show that the SICM method allows for a deep characterization of the cell-soft hydrogel system, with the substrate stiffness being lower than that of the cells, and that the self-assembling Fmoc-FF hydrogel would be a promising biocompatible scaffold for these cells, then this method could be used in the future to study various epithelial/neural or other cell types. This includes investigating their mechanical properties after treatment with drugs, growth factors, or hydrogel degradation. These considerations were added to the Introduction section.

Comments 6: This research assessed the mechanical properties of MCF-7 malignant breast cancer cells but it need also to test benign breast cells as negative control (e.g. MCF10A breast cell lines).

Response 6:

As previously noted, MCF-7 breast cancer cells were used in this study not for the investigation of breast cancer malignancies, but as a convenient and well-researched model. Their biocompatibility with the self-assembling Fmoc-FF hydrogel can be considered a reference point, against which the potential use of this gel with other, more demanding cell types can be evaluated. These considerations were added to the Introduction section.

Comments 7: Figure 2 (line 155). The lateral scale bar needs to be furnish for the images displayed in all the panels.

Response 7:

The scale bar was added to all the images in Figure 2.

Comments 8: “The resolution reduction of AFM data (…) higher values of applied forces and higher values of indentation (…) 1500 nm in AFM experiments (…) 220 nm in SICM studies” (liens 211-215). The elastic modulus obtention directly relies on the exerted load forces and the indentation depths. These values are required to be consistent among AFM ans SICM experiments in order to make comparable the data gathered by both techniques.

Response 8:

One of the main advantages of SICM over AFM is its noninvasiveness, i.e., the absence of direct capillary impact on cells. Therefore, using the same loading force and indentation depth for both experiments would make it pointless to use the SICM method to study the living cell-hydrogel system.

On the other hand, unfortunately, it was not feasible to achieve smaller indentations with AFM for very soft samples such as Fmoc-FF gels and cells used here. The trigger force applied in the force volume mode should be large enough to prevent false contact and provide stable force mapping. The problem can’t be solved with the usage of softer cantilevers, since they are more affected by

the environmental noises and hydrodynamic drag during the force mapping. Therefore, the lowest possible trigger force (around 0.5-1 nN) resulted in indentations around 500-1500 nm.

Comments 9: Materials & Methods. “Chemical structure (Fig. S1 (A)) and FTIR analysis (…)” (line 385). The full-name needs to be stated the first time that a term appears in the text. Then, the abbreviation should be placed between brackets. This comment should be taken into account for the rest of the main manuscript body text.

Response 9:

This inaccuracy has been corrected for all terms in the text.

Comments 10: “(…) υ is the Poisson ration of the sample (assumed to be 0,5 for the cells and hydrogel)”. (lines 473-474). Why did the authors select a Poisson ratio of 0.5 instead of lower values (e.g. 0.30 or 0.35)? A brief explanation should be furnished in this regard.

Response 10:

A Poisson ratio of 0.5 is generally selected for both hydrogels [Ahearne 2005] and cells [Efremov 2019, Harris 2011], as it was shown that they maintain their volume under load. A brief explanation and references were added into the text.

-Ahearne, M., Yang, Y., El Haj, A. J., Then, K. Y., & Liu, K. K. (2005). Characterizing the viscoelastic properties of thin hydrogel-based constructs for tissue engineering applications. *Journal of the Royal Society Interface*, *2*(5), 455-463.

-Efremov, Y. M., Velay-Lizancos, M., Weaver, C. J., Athamneh, A. I., Zavattieri, P. D., Suter, D. M., & Raman, A. (2019). Anisotropy vs isotropy in living cell indentation with AFM. *Scientific reports*, *9*(1), 5757.

-Harris, A. R., & Charras, G. T. (2011). Experimental validation of atomic force microscopy-based cell elasticity measurements. *Nanotechnology*, *22*(34), 345102.

Comments 11: “5. Conclusions” (lines 525-537). This section perfectly remarks the most relevant outcomes found by the authors in this work and also the promising future prospectives. The authors should furnish a brief statement to discuss about the future action lines to pursue the topic covered in this work.

Response 11:

The section “Conclusions” were was expanded, further plans for work in this area were described, please see the manuscript: “In the future, it is planned to modify the composition of the gel to make it more suitable as a biocompatible scaffold for living cells. This could be achieved, for example, by adding other peptides such as Fmoc-F5 or PEG8-(FY)3 hexapeptide [Diaferia 2019]. Subsequently, specific tissue types can be targeted by using epithelial or neuronal cells (depending on the task at hand). This will allow for the dynamic study of changes in cell metabolism when adhered to the surface of self-assembling gels, the effects of growth factors and other substances

on the systems under investigation, and simultaneously, the degradation of the gel itself using the SICM method".

- Diaferia, C.; Ghosh, M.; Sibillano, T.; Gallo, E.; Stornaiuolo, M.; Giannini, C.; Morelli, G.; Adler-Abramovich, L.; Accardo, A. Fmoc-FF and hexapeptide-based multicomponent hydrogels as scaffold materials. *Soft Matter* **2019**, *15(3)*, 487-496.