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Magnetic tweezers in a microplate format

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TITLE:

Magnetic Tweezers in a Microplate Format

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KEYWORDS:

mechanobiology, tweezers, 3D-printed, magnets, FRET, ensemble

SUMMARY:

Here, we describe the use of a novel microplate assay to enable mechanical manipulation of biomolecules while performing ensemble biochemical assays. This is achieved using a microplate lid modified with magnets to create multiple static magnetic tweezers across the microplate.

ABSTRACT:

Mechanobiology describes how the physical forces and mechanical properties of biological material contribute to physiology and disease. Typically, these approaches are limited single-molecule methods, which restricts their availability. To address this need, a microplate assay was developed that enables mechanical manipulation while performing standard biochemical assays. This is achieved using magnets incorporated into a microplate lid to create multiple magnetic tweezers. In this format, force is exerted across biomolecules connected to paramagnetic beads, equivalent to a typical magnetic tweezer. The study demonstrates the application of this tool with FRET-based assays to monitor protein conformations. However, this approach is widely applicable to different biological systems ranging from measuring enzymatic activity through to the activation of signaling pathways in live cells.

INTRODUCTION:

Mechanobiology focuses on understanding how the propagation of physical forces within and between cells regulates cellular activity[1,2] and how this correlates with the organization and dynamics of both proteins and cells.

Single-molecule force measurements have revealed how force is used in biological systems, from single proteins to whole cells and tissues[3–7]. These challenging experiments require specialized equipment and technical expertise. Conversely, standard biochemical assays can be performed at higher throughput in readily available commercial equipment.

Here, the study describes a mechanobiology assay that enables magnetic tweezer-based manipulation and biochemical assays to be performed together[8]. Magnets are placed on a 3D printed microplate lid (**Figure 1A–D**), enabling the use of commercial plate readers for the assays. Force is applied across the biomolecule of interest by coupling the molecule to paramagnetic particles. The magnets then exert tension across the molecule. Altering the distance between the particles and magnets adjusts the exert force across the biomolecule (**Figure 1E**).

We represent the use of this assay using the actin-based molecular motor, Myosin VI. Myosin VI is regulated by intramolecular backfolding[9]. Myosin VI has been shown to exist in an auto-inhibited state, whereby the binding of partner proteins, such as NDP52, triggers the unfolding of myosin VI[10,11]. To perform these assays, we will use a dual-labeled construct of the myosin VI tail domain with an N-terminal GFP and a C-Terminal RFP whereby backfolding of the protein generates Fluorescence Resonance Energy Transfer (FRET) between GFP and RFP. The N-terminus also carries a biotinylation tag to immobilize the protein on the surface. We use this assay in combination with FRET measurements to show how force can impact myosin VI back-folding.

PROTOCOL:

Sample proteins required for this experiment and a list of reagents are found in the **Table of Materials**. Equivalent proteins should be produced for the user's system of study to measure conformation changes.

1. 3D printed magnetic lid

1.1. Design a microplate lid to house the magnets within a 24-well microplate. An example CAD file can be downloaded from GitHub[12]. The measurements are shown in **Figure 1**.

NOTE: The height of the pedestal can be altered to change the distance between magnets and surface to alter the applied force. In addition, the final force exerted upon the particle depends on the particle size and gap between the pair of magnets. Equations to calculate the applied force for 1 µm and 2.8 µm particles at 0.5 mm, 1 mm, and 2 mm gaps are given in Dos Santos et al.[8]. For the experiments described here with 2.8 µm particles and a 2 mm gap, use Equation 1.

Equation 1: $$FORCE = -0.0078 + 43.5^{\left(-\frac{(Z-0.4)}{1.58}\right)} - 30.4^{\left(-\frac{(Z-0.4)}{0.319}\right)}$$

1.2. 3D print a microplate lid using Polyethylene Terephthalate Glycol (PETG) on a 3D printer system.

NOTE: Use a printer with a layer resolution of 0.02 mm to enable accuracy and reproducibility. The impact of printer accuracy was discussed in Dos Santos et al.[8].

1.3. Attach pairs of 5 mm cube Neodymium N42 magnets using adhesive to the pedestals with the magnetic poles orientated to create the field directed towards the bottom of the microplate.

1.4. Leave at least 1 position empty to act as a no-magnet control.

1.5. Store in a sealed container of sufficient size to readily place and remove the lid.

2. Microplate surface modification

2.1. Take a clean non-treated glass-bottomed 24-well microplate.

2.2. Wash the wells 3x with 500 µL of wash buffer (50 mM Tris-HCl (pH 7.5) and 50 mM NaCl) at room temperature.

2.3. Add 200 µL of biotin-BSA (0.2 mg/mL) in the wash buffer and leave it undisturbed for 15 min at room temperature.

NOTE: Biotin-BSA is used for both passivation and attachment. Other passivation and surface attachment strategies can be used. For first-time use and optimization, the concentration of biotin-BSA can be varied up to 1 mg/mL.

2.4. Wash the wells 3x with 500 µL of wash buffer.

2.5. Add 200 µL of streptavidin (20 µg/mL) in the wash buffer and leave it undisturbed for 15 min at room temperature.

NOTE: For first-time use, the concentration of streptavidin can be varied up to 1 mg/mL.

2.6. Remove the solution and then wash the wells 3x with wash buffer.

2.7. Cover the wells with 500 µL of wash buffer.

2.8. Store at 4 °C until ready to use. Plates should be used on the same day.

3. Sample preparation: Protein immobilization

3.1. Allow the surface-modified microplate to reach room temperature.

3.2. Wash the wells with 500 µL of wash buffer.

3.3. Add 100 nM biotin-eGFP-Myosin VI Tail-mRFP (stock sample protein) and incubate for 15 min at room temperature in wash buffer.

NOTE: This example protein is produced as described by Dos Santos et al.[8]. Concentrations and incubations times can vary depending upon the protein.

3.4. Wash the wells 3x with 500 µL of wash buffer.

3.5. Set up the plate reader to record fluorescence spectra for GFP (excitation 490 nm and emission 510–600 nm) and RFP (excitation 560 nm and emission 580–650nm) to confirm the presence of the fusion proteins.

3.6. If there is a lack of protein, increase the concentration at step 3.3.

NOTE: Poor binding could relate to inadequate biotin-BSA and/or streptavidin binding, and therefore, the amounts could be increased 10-fold.

3.7. If possible, perform a well-scan measurement to determine if the fusion protein is bound across the microplate (**Figure 2**).

NOTE: This measurement will record the fluorescence intensity at specific points across the microplate well. It will reveal if the protein is bound across the well.

3.8. Ensure that the signal is uniform (within 5%) in the center of the well where the assay measurement occurs.

4. Magnetic bead preparation

4.1. Vortex the vial of 2.8 µm paramagnetic beads with recombinant Protein A (**Table of Materials**) for 30 s to resuspend the beads.

4.2. Transfer the volume corresponding to 1 mg of paramagnetic beads to a 1.5 mL tube.

4.3. Place the tube in a magnetic isolator (**Table of Materials**) and then remove the supernatant.

4.4. Resuspend the beads in 200 µL of PBS by gently pipetting the solution up and down.

4.5. Place the tube in the magnetic isolator and then remove the supernatant.

4.6. Repeat the wash step 3x.

4.7. Dilute 10 µg of Anti-RFP antibody in 200 µL of PBS.

4.8. Isolate the beads in the magnetic isolator and then resuspend in the antibody solution.

4.9. Rotate at 20 rpm for 10 min at room temperature on a benchtop rotator.

4.10. Place the tube in the magnetic isolator and remove the supernatant.

4.11. Wash 3x in PBS.

4.12. Resuspend the beads in 200 µL of wash buffer.

4.13. To confirm antibody immobilization on the beads, incubate the antibody-loaded beads with a solution of >1 µM RFP fusion protein.

NOTE: Confirm visually whether the protein is isolated by the beads.

4.14. Alternatively, measure the fluorescence intensity of the supernatant (excitation 560 nm and emission 580–650nm) for RFP in a fluorescence spectrometer.

5. Sample preparation: Bead attachment

5.1. Add 10 µg of the paramagnetic bead-antibody fusion diluted in wash buffer (200 µL) to the protein-bound microplate wells.

5.2. Do not place beads in one control well to check for the effect of beads on the fluorescent signal. Prepare a control sample without antibodies to determine any non-specific interactions with the beads.

5.3. Incubate the microplate at room temperature for 30 min.

5.4. Wash the wells 3x with 500 µL of wash buffer.

5.5. If available locally, check that the beads are bound to the surface on a brightfield microscope. If there is a lack of beads, increase the concentration in step 5.1 to 500 µg/mL.

6. Data acquisition

6.1. Place the microplate, without lid, into a fluorescent plate reader at 25 °C.

6.2. Set up the plate reader to record fluorescence spectra for GFP (excitation 490 nm and emission 510–600 nm) and RFP (excitation 560 nm and emission 580–650 nm) to confirm the presence of the fusion proteins.

NOTE: Measure/record the fluorescence spectra through the bottom of the microplate to place the lid on top. Therefore, a suitable microplate reader should be selected.

6.3. Record a FRET fluorescence spectrum (excitation 490 nm and emission 510–650nm), or monitor fluorescence at 510 nm and 610 nm with excitation at 490 nm. This is set up according

to the manufacturer's guidance.

6.4. Remove the microplate from the reader and add the magnetic lid.

6.5. Return to the plate reader and leave the sample for 2 min.

6.6. Repeat the FRET measurement, as in step 6.3.

7. Data analysis

7.1. Export the data for import into a spreadsheet (e.g., Excel).

7.2. Manually extract the data for Donor GFP at 510 nm and acceptor RFP at 610 nm.

7.3. Calculate relative FRET using Equation 2 for each condition.

Equation 2: $FRET = \frac{A}{(D+A)}$

A: acceptor intensity (RFP 610 nm), D: donor intensity (GFP 510 nm).

NOTE: Use the recombinant RFP under the same excitation and emission conditions to control for the background excitation of the acceptor.

REPRESENTATIVE RESULTS:

Figure 2 shows an example of a well-scan measurement where the fluorescence intensity of GFP has been recorded at 1 mm intervals across the microplate well. Typical fluorescence measurements are performed at the center position of the microplate well (position 8,8 in **Figure 2**); it is, therefore, important that there is bound protein at this location. As shown in **Figure 2**, the intensity is highest in the center of the well within a radius of a few millimeters. Typically, immobilization is better away from the edges of the well, potentially due to protein binding to the side walls. The poor signal due to lack of protein binding or defects in biotin-BSA and/or streptavidin would be identified here. A fluorescent streptavidin conjugate could be used to confirm biotin-BSA on the surface. Likewise, a control protein consisting of biotin-GFP would determine if streptavidin is bound to the biotin-BSA surface. As mentioned in the protocol, the amount of biotin-BSA, streptavidin, and protein can be increased.

Figure 3 provides example data for the forced-induced unfolding of myosin VI. The biotin-eGFP-Myosin VI Tail-mRFP construct[11] was immobilized on the microplate surface and coupled to the paramagnetic beads using an anti-RFP antibody (**Figure 3A**). In this scenario, a high FRET state is found when myosin VI is backfolded where the fluorescent proteins are in close proximity.

A FRET spectrum was recorded in the absence of the magnetic lid (**Figure 3B**), where a signal at 610 nm can be observed. If no signal is seen at either 510 nm or 610 nm, individual fluorescence

spectra should be recorded for GFP and RFP to determine if the fusion is intact. The experiment was then repeated in the presence of the lid with magnets generating an assumed force of 1.8 pN, arising from a 0.5 mm pedestal with a 2 mm gap between magnets. The intensity at 610 nm was lost, indicating a loss of FRET due to a conformation change. The relative FRET was calculated using Equation 1, where a decrease is observed following the addition of force. A change in conformation triggering the FRET change will lead to an intensity decrease in 610 nm (acceptor) and an intensity increase in 510 nm (donor). If only one changes, then the effect is likely to be the disruption of the fluorescent proteins rather than the conformation change.

The experiment was repeated over a range of forces where magnets were positioned at different heights relative to the bottom of the microplate. Based on the height, the gap between magnets, and particle size, the force applied to the sample (assumed force) can be calculated based on equations[8]. The Relative FRET decreased upon the addition of force up to 1 pN, but it did not change for greater forces (**Figure 3C**). The absence of anti-RFP antibodies or paramagnetic beads stops the force application upon myosin VI, and therefore there is no change in Relative FRET. The presence of the antibody or beads could cause a change in the fluorescence signal due to environmental changes[13]. This may impact the FRET calculation, so it is critical to perform the control experiments. Lastly, the application of high force could remove the construct from the surface or cause a break in the protein. Therefore, it is important to ensure there is still a signal from both GFP and RFP following a decrease in Relative FRET.

FIGURE AND TABLE LEGENDS:

Figure 1: Example design for 3D printed magnetic lid. (**A**) Side and (**B**) top-down projections showing the position of the pedestals which hold the magnets. The pedestals in rows 1 and 2 are at 0.5 cm, 0.4 cm, 0.3 cm, 0.2 cm and 0.1 cm, respectively. The measurements stated here will differ for each brand and type of microplate. (**C**) The pedestals determine the force applied to the sample. The width of the divider (black) can be altered to change the space between the magnets. This will alter the strength of the magnetic field, as detailed in Dos Santos et al.[8]. The height of the spacer (blue) can be varied to adjust the distance between the magnets and the surface to alter the force applied on the sample, as described in Dos Santos et al.[8]. (**D**) Image of an example 3D printed lid. (**E**) Principle for exerting forces on biomolecules. A molecule of interest is attached to the surface and bound to a paramagnetic particle. A pair of 5 mm cube neodymium magnets attached to the lid (grey) exert force on the beads through their magnetic field. Increasing the proximity of the magnets to the beads leads to higher forces exerted across the molecule. In this assay, the proximity is altered using different size pedestals.

Figure 2: Well scan depicting bound protein on the surface of the microplate. The eGFP-Myosin VI Tail-mRFP construct was immobilized on the surface through an N-terminal biotinylation tag, as shown in **Figure 3A**. A well-scan measurement was performed across the microplate well at 1 mm intervals monitoring the fluorescence of GFP (excitation at 490 nm and emission at 515 nm). The GFP fluorescence intensity numbers are presented as a heat plot.

Figure 3: Representative results for the force-induced unfolding of myosin VI. (**A**) Diagram of the assay format. The eGFP-Myosin VI Tail-mRFP construct was immobilized through an N-

terminal biotinylation tag (BRS). The C-terminus was coupled to Protein-A paramagnetic beads through an anti-RFP. When no force is applied, the protein is folded, which generates a high FRET signal. The application of force (*F*) triggers unfolding, leading to a low FRET state. (**B**) Example fluorescence spectra following GFP excitation in the absence (red) and presence of 1.8 pN of force (dark blue). INSET: Relative FRET calculated using Equation 1 for 3 replicates. The color legend matches the spectra. (**C**) Plot of Relative FRET obtained under different forces by varying the pedestal height from 0.1–1 cm. Data are also shown for control experiments in the absence of the antibody and beads. Error bars represent SEM from 3 independent experiments.

DISCUSSION:

This approach enables force-based measurements to be readily applied in a microplate using fluorescent plate readers. Importantly, this assay format assumes there is functional protein when it is bound to a surface. Therefore, prior knowledge is required before embarking on these measurements to ensure there is protein activity. It is also beneficial to make sure that the binding of molecules to the paramagnetic beads and surface is optimized for each system.

This concept can be modified to function across all types of microplates; however, the magnet selection will need to be adapted for microplates above 24 wells, as magnets cannot fit in the wells. Magnets can still be placed on top of the wells; however, in a 24 well plate, this does not lead to stray fields across the other wells[8]; in smaller wells, such as 96 well plates, there may be field variations between the wells. Lastly, it is possible to use the same approach while performing fluorescence microscopy on an inverted microscope.

This approach does not have the force accuracy found within single-molecule measurements and therefore aims to complement those methodologies. Furthermore, this approach applies a fixed amount of force to the sample and does not allow forces to be varied within a well or monitor forces exerted by a protein. Moreover, assumptions are made which does not apply to single-molecule measurements. For example, we cannot determine the number of molecules attached to each bead. However, the molecule attachment is assumed to be the same for each bead-tether pairing; therefore, relative force changes can be measured. In addition, we cannot determine if a molecule is bound by a paramagnetic particle, and therefore we do not know what fraction of the biomolecule population is generating the signal.

In summary, using this approach, it is now possible to quantitatively study biological processes using established assays under mechanical load. Moreover, multiple conditions can be tested simultaneously, thereby increasing the throughput.

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DISCLOSURES:

The authors declare no competing interests.

REFERENCES:

1. Jansen, K. A. et al. A guide to mechanobiology: Where biology and physics meet. *Biochimica et Biophysica Acta*. **1853**, 3043–3052 (2015).
2. Dos Santos, A., Toseland, C. P. Regulation of nuclear mechanics and the impact on dna damage. *International Journal of Molecular Sciences.* **22** (6), 3178 (2021).
3. Dos Santos, A. et al. DNA damage alters nuclear mechanics through chromatin reorganization. *Nucleic Acids Research*. **49** (1), 340–353 (2020).
4. Elosegui-Artola, A. et al. Force triggers YAP nuclear entry by regulating transport across nuclear pores. *Cell.* **171**, 1397–1410 e1314, (2017).
5. Lherbette, M. et al. Atomic force microscopy micro-rheology reveals large structural inhomogeneities in single cell-nuclei. *Scientific Reports.* **7**, 8116 (2017).
6. Seo, D. et al. A mechanogenetic toolkit for interrogating cell signaling in space and time. *Cell.* **165**, 1507–1518 (2016).
7. Yao, M. et al. The mechanical response of talin. *Nature Communications* **7**, 11966 (2016).
8. Dos Santos, A., Fili, N., Pearson, D. S., Hari-Gupta, Y., Toseland, C. P. High-throughput mechanobiology: Force modulation of ensemble biochemical and cell-based assays. *Biophysical Journal.* **120**, 631–641 (2021).
9. Fili, N., Toseland, C. P. Unconventional myosins: How regulation meets function. *International Journal of Molecular Sciences.* **21** (1), 67 (2019).
10. Fili, N. et al. Competition between two high- and low-affinity protein-binding sites in myosin VI controls its cellular function. *Journal of Biological Chemistry.* **295**, 337–347 (2020).
11. Fili, N. et al. NDP52 activates nuclear myosin VI to enhance RNA polymerase II transcription. *Nature Communications.* **8**, 1871 (2017).
12. MagPlate at GitHub <https://github.com/cptoseland/MagPlate> (2021)
13. Toseland, C. P. Fluorescent labeling and modification of proteins. *Journal of Chemical Biology.* **6**, 85–95 (2013).

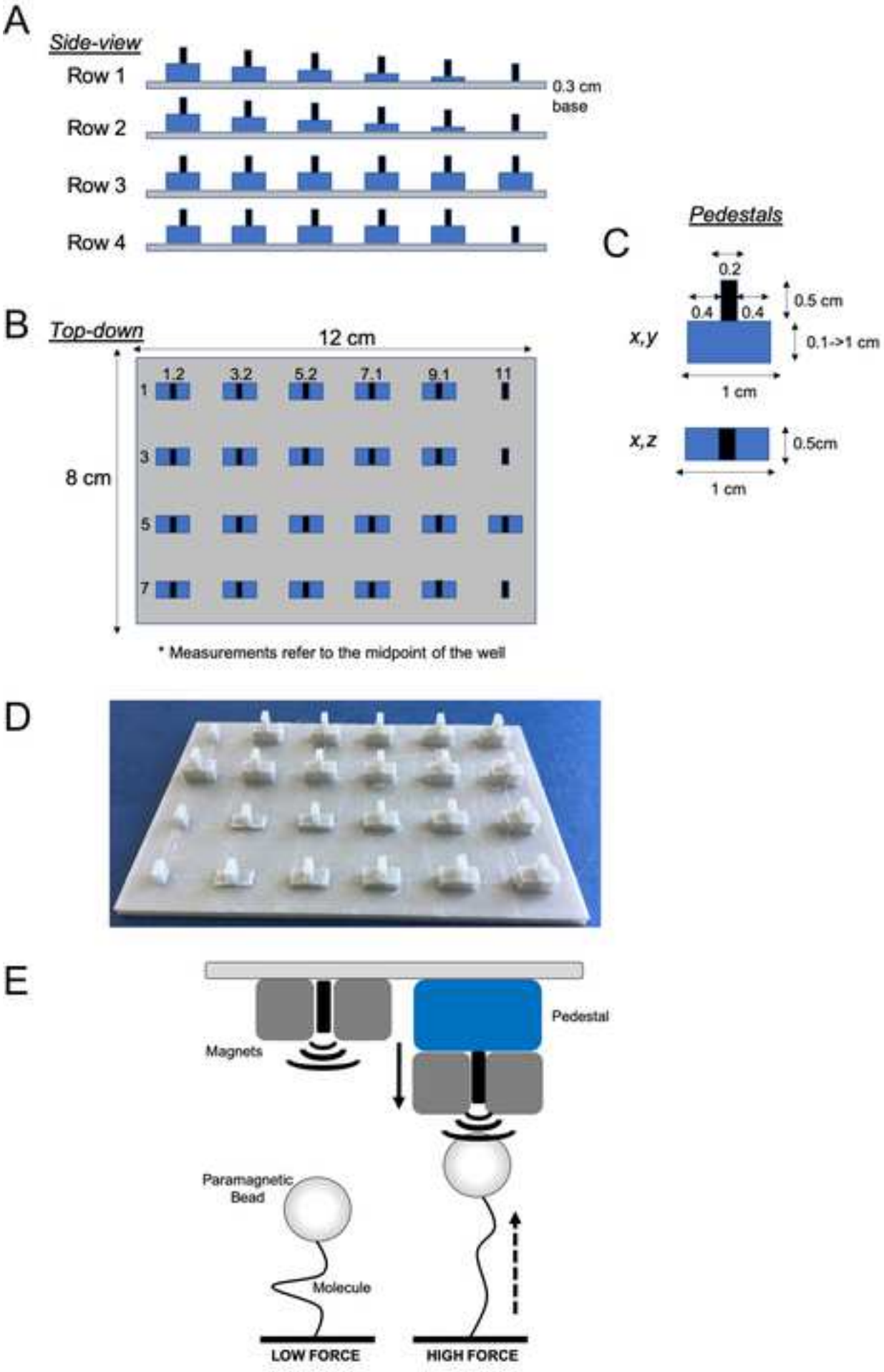
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Figure2

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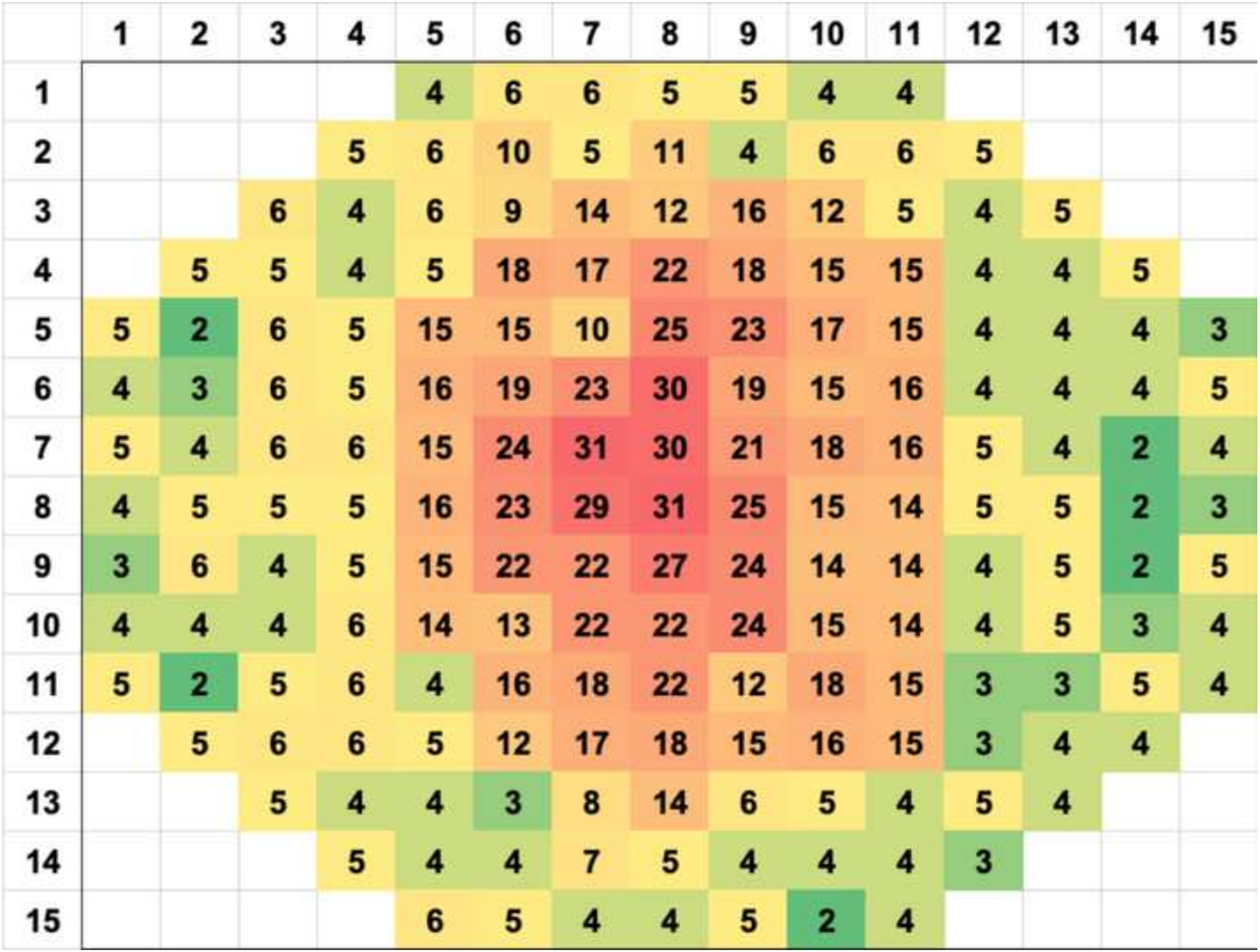

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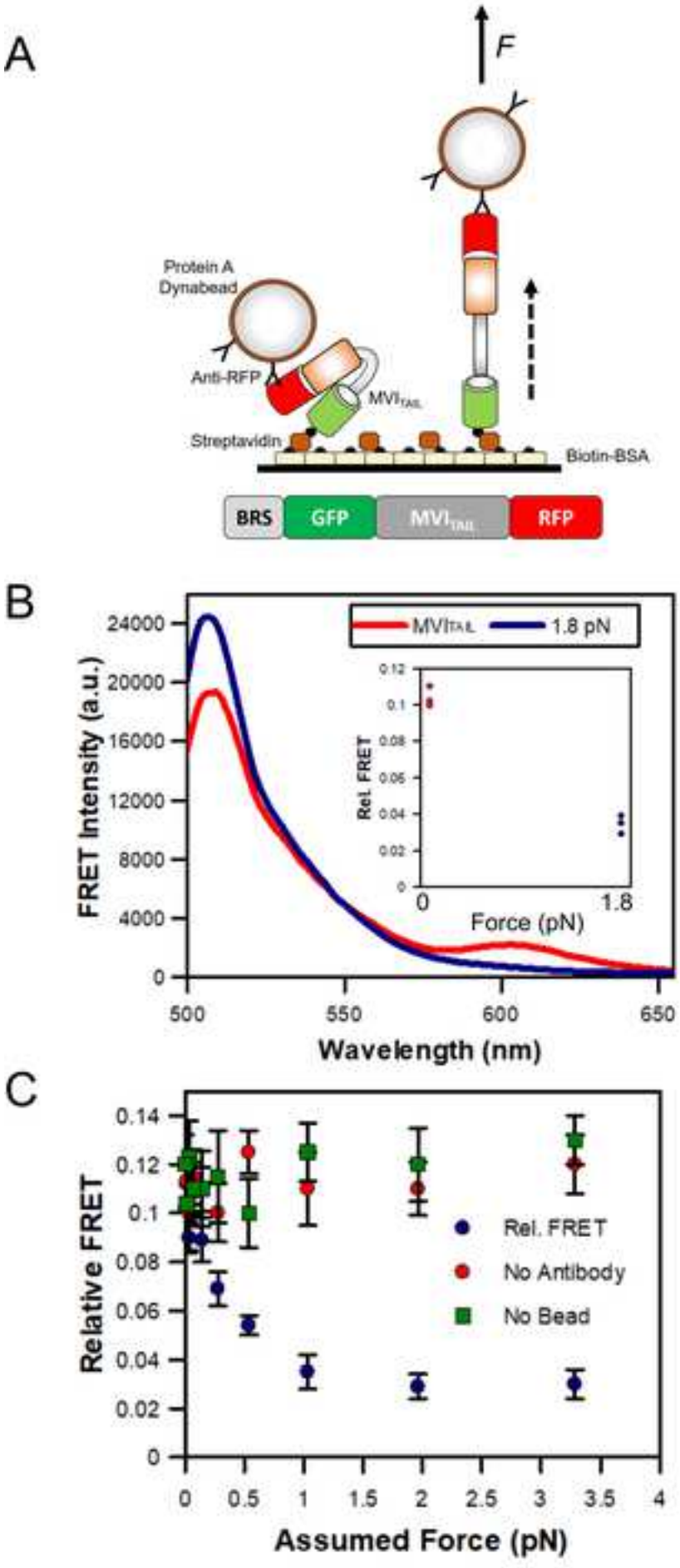

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We have addressed these comments using track changes.

2. Please revise the lines to avoid the issue of plagiarism: 47-49, 246, 307-308, 323-324. Please refer to the iThenticate report attached.

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3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Dynabeads" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

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4. Figure 3: Please add the title of the X-axis in the inset graph (3B).

This was already added to the Figure – as stated by “Force (pN)” – we have uploaded the figure again.

5. Please ensure that the Table of Materials includes all the essential items (consumables, reagents, chemicals, equipment, etc.) used in this study.

Further details have been added to the table.