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Characterisation of cuticle mechanical properties: analysing stiffness in layered living systems to understand surface buckling patterns

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Development of a living organism is a highly regulated process during which biological materials undergo constant change. *De novo* material synthesis and genetically-regulated changes in mechanical properties of materials are key for organ development. However, few studies have attempted to produce quantitative measurements of the mechanical properties of biological materials during growth. Such quantitative analysis is particularly challenging where the material is layered, and yet layering of materials with different mechanical properties may be essential to morphogenetic pattern formation. This is the case for the *Hibiscus trionum* flower petal, where buckling of the cuticle on top of the epidermal cell wall forms ridges, producing an iridescent effect. This ridge formation is hypothesised to be due to mechanical instability, which directly depends upon the mechanical properties of the individual layers of cuticle and cell wall. We set out to develop methods to measure the mechanical properties of the surface layers of plant epidermal cells through atomic force microscopy (AFM). To ensure that our results were reproducible and represented the most appropriate combination of experimental parameters, we used the uniaxial tensile tester for ultrathin films (TUTTUT) to provide independent measurement of cuticle stiffness. We explored mechanical properties of the upper cuticle and lower cuticular layer of the epidermal cell surface across growth stages. In addition to offering technical approaches to explore the stiffness of living layered materials, our findings suggest that temporal changes in biological material properties are key to understanding the development of biological surface patterns.

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Introduction

Hibiscus trionum petals display beautiful colours, which are produced by pigments and structural features. In the purple pigmented regions, the cuticle presents elongated rows of nano-ridges on top of semi-flat cells, which form a semi-ordered diffraction grating.¹ The diffraction of the light by these ridges creates a weak iridescence with a scattered blue halo effect that is perceived by bees and can increase the salience of the flowers to foraging pollinators.² The petal surface forms a bilayer structure, with the extracellular cuticle

proper (composed primarily of cutin and waxes) sitting on top of a cell wall-based cuticular layer (composed of cellulose impregnated with cutin monomers). It has been shown using dissected petal samples combined with continuum mechanical theories that mechanical buckling is sufficient to explain the formation of the *Hibiscus trionum* petal cuticular striations.^{3,4}

We have recently explored the theoretical basis of mechanical buckling in the *Hibiscus trionum* system.⁴ According to linear elastic buckling theory, a bilayer system with a stiff thin film resting on a soft substrate will form a periodic wrinkle pattern when an in-plane compressive strain exceeds a critical threshold value, ϵ_c . If the substrate thickness is much larger than the film thickness, the wavelength, λ , at the onset of a wrinkled pattern in an incompressible material is determined by:⁵

$$\lambda = 2\pi h \left(\frac{E_f}{3E_s} \right)^{\frac{1}{3}} \quad (1)$$

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In addition to a need to understand living bilayers, knowledge of how the mechanical properties of materials change during growth would aid our understanding of how specific wrinkle patterns and dimensions are achieved during petal development. While it seems intuitively likely that the mechanical properties of biological materials change during

In this work, we developed tools to measure the mechanical properties of the cuticle proper (the surface layer) and the cuticular layer (the substrate layer) on the surface of petals from the onset of surface buckling to the fully developed flower. We used two independent methods, atomic force microscopy (AFM) and the uniaxial tensile tester for ultrathin films (TUTTUT). AFM is accessible to many biology labs, while TUTTUT is harder to access, but the use of the two techniques, in separate labs with separate experimentalists (and on different continents), allowed us to explore the reproducibility of our AFM data and provide confidence in our findings. By directly characterising the material and geometric properties of *H. trionum* petals, we were able to assess whether the morphology we observe can be predicted by mechanical theory. We found that the properties of the tissue at the start of buckling can predict the wrinkling initiation strain and the wrinkle wavelength. We also found that the pattern wavelength is set immediately following initiation, such that the later development of the petal does not substantially change the pattern wavelength, perhaps due to the inelasticity of biological materials during growth. The introduction of TUTTUT further provides pathways for exploring the cuticle mechanical response at larger strains, beyond where AFM characterisation is appropriate. The approach described here for measurement of mechanical properties of a living bilayer during development will provide new insights in the field of biomechanics and facilitate greater understanding of biological pattern formation.

An AFM JPK NanoWizard with a cantilever tip of tetrahedral shape (ATEC-CONT Au10 S/N: 74454F6L1180 Nanosensor; nominal values: length 450 μm , mean width 50 μm , thickness 2 μm ; Fig. S1) was used. This tip was chosen because of its small half cone angle that increases its performance on samples with a small pattern size combined with steep sample features. Three cantilevers were used over the course of the experiments whose

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by hand under the microscope. The sections were stained with Toluidine blue (0.05%) for 1 minute, then washed with 1X PBS for 1 minute. Bright field pictures were taken with a VHX-5000 Keyence microscope.

Characterisation of growth of *Hibiscus trionum* petal layers

We followed the protocol described in ref. 19 to conduct the uniaxial tensile tester for UltraThin films (TUTTUT) to measure the force-displacement responses of the cuticle. The TUTTUT cantilevers were calibrated before each set of measurements, following protocols previously published.¹⁹ We floated the cuticle films on a water bath. A silicon wafer was dropped on the grip section of the film. The water level was lowered, and the wafer positioned into the clamp and rigidly fixed onto the walls of the bath container. The other side of the cuticle film was attached to a cantilever (an aluminium-coated cover glass). The grip section wafer was set to be parallel to the bottom edge of the cantilever. The stage connected to the water bath container was then pulled at a constant velocity of 0.2 mm s^{-1} . A laser reflective system recorded the cantilever deformation, which was then used to calculate the resulting force on the cuticle film. For each petal developmental stage, we analysed three isolated cuticle films. We used the force-displacement responses to determine the average stress-strain responses of the cuticle samples. The strain was calculated by dividing the applied far-field displacement by the initial sample length. Since the shapes of the isolated cuticle samples were irregular (*i.e.*, not rectangular nor standard geometries, such as dog bone shapes), we used finite element analysis (FEA) with the same geometries as the experiments to calibrate a geometric factor for each experiment to determine the Young's modulus of the cuticles (Fig. S5). Using FEA to calibrate the stress-strain response has been used previously for inhomogeneous deformation fields.²⁰ Specifically, the Young's modulus of the FEA model was adjusted until the relationship between resultant force and applied displacement matched experimentally measured trends for each sample geometry. We then obtained the nominal cross-sectional area in the loading direction A by $A = kL/E$, where k is the experimentally measured stiffness, L is the gauge distance between load grippers, and E is the determined Young's modulus. The stress was then determined by dividing the resultant force by the nominal cross-sectional area, F/A .

To assess the effect of removal of cuticle proper on integrity of the cuticular layer below, the cuticle proper was removed from the petals of stage 5 flowers using a sharp, flexible razor blade under the microscope. Petals with intact cuticle proper were included as controls. Samples were infiltrated with 1X PBS and embedded in 4% agarose. Thin cross-sections were generated

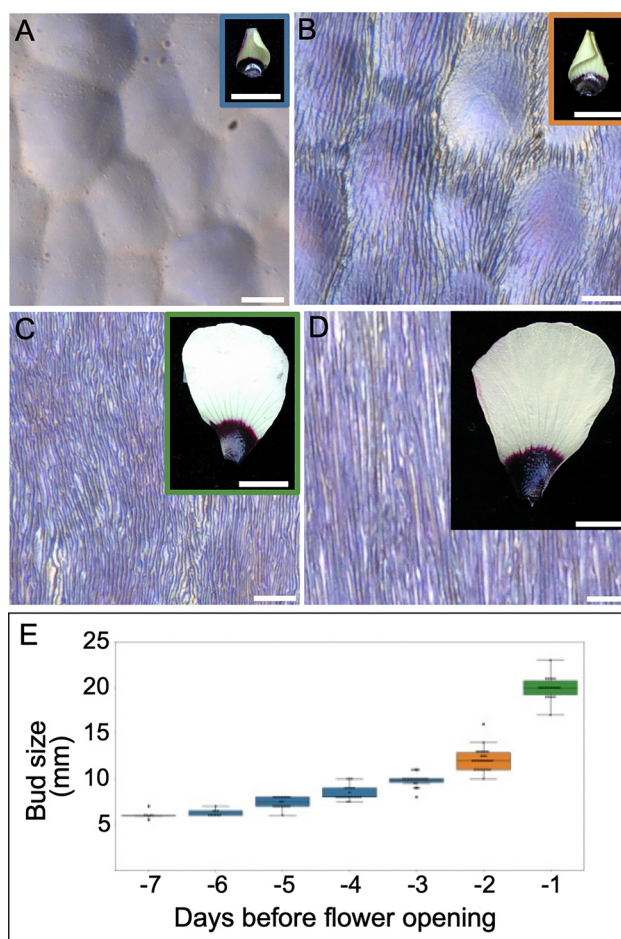
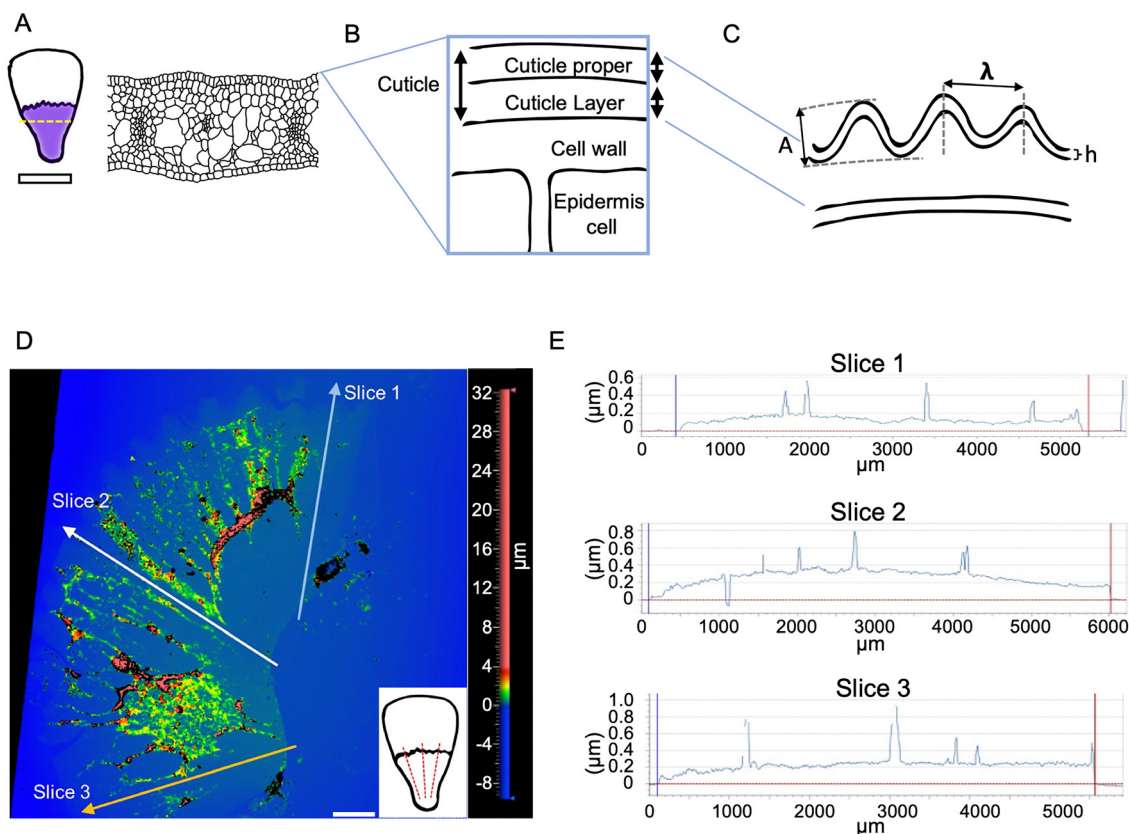


Fig. 1 Petal growth and striation formation in *Hibiscus trionum*. (A) A 10 mm flower bud petal with smooth petal epidermis (pre-striation stage). (B) 12 mm flower bud petal with striations starting to form. (C) Mature flower bud petal with striations formed across the purple area of the petal. (D) Open flower petal with fully developed semi-ordered striations. (E) Flower bud size (without epicalyx) from 7 days before opening. Pre-striation stages (–7 to –3 days) are indicated in blue bars, the striation onset stage (–2 days) in orange, and the mature bud stage in green; these colours indicate stage in the petal images in A–D. Scale bars represent 10 μ m in A–D, and 10 mm in the insets.

A number of papers have suggested that the cuticular striations of *H. trionum* develop as a result of mechanical instability,^{22,23} with our latest model⁴ proposing that their formation is the result of differences in the mechanical properties of two layers



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All of our measurements indicate that the cuticular layer (substrate) is softer than the cuticle proper (upper layer), as hypothesized in previous studies.^{3,4,25} The difference in the stiffness between these two layers increases through developmental time, as the cuticular layer becomes softer during petal growth to a much greater degree (approximately 10-fold) than does the cuticle proper (approximately 1.3 to 2.5-fold, depending on method of measurement).

The predicted wavelengths we obtained were similar to those that we measured (Table 1), and within the range of variation seen in nature. However, at the open flower stage the differences between theoretical and observed wavelengths were more significant than at the earlier flower stages.

Here, we analysed stiffness of a layered living tissue during development to understand the formation of striation patterns on the epidermal surface in *H. trionum* petals. We describe AFM and TUTTUT techniques, which generate reasonably consistent data, providing confidence in the methods applied. We propose that our AFM protocol, using morphology-informed indentation depths and mechanical removal of upper layers, is

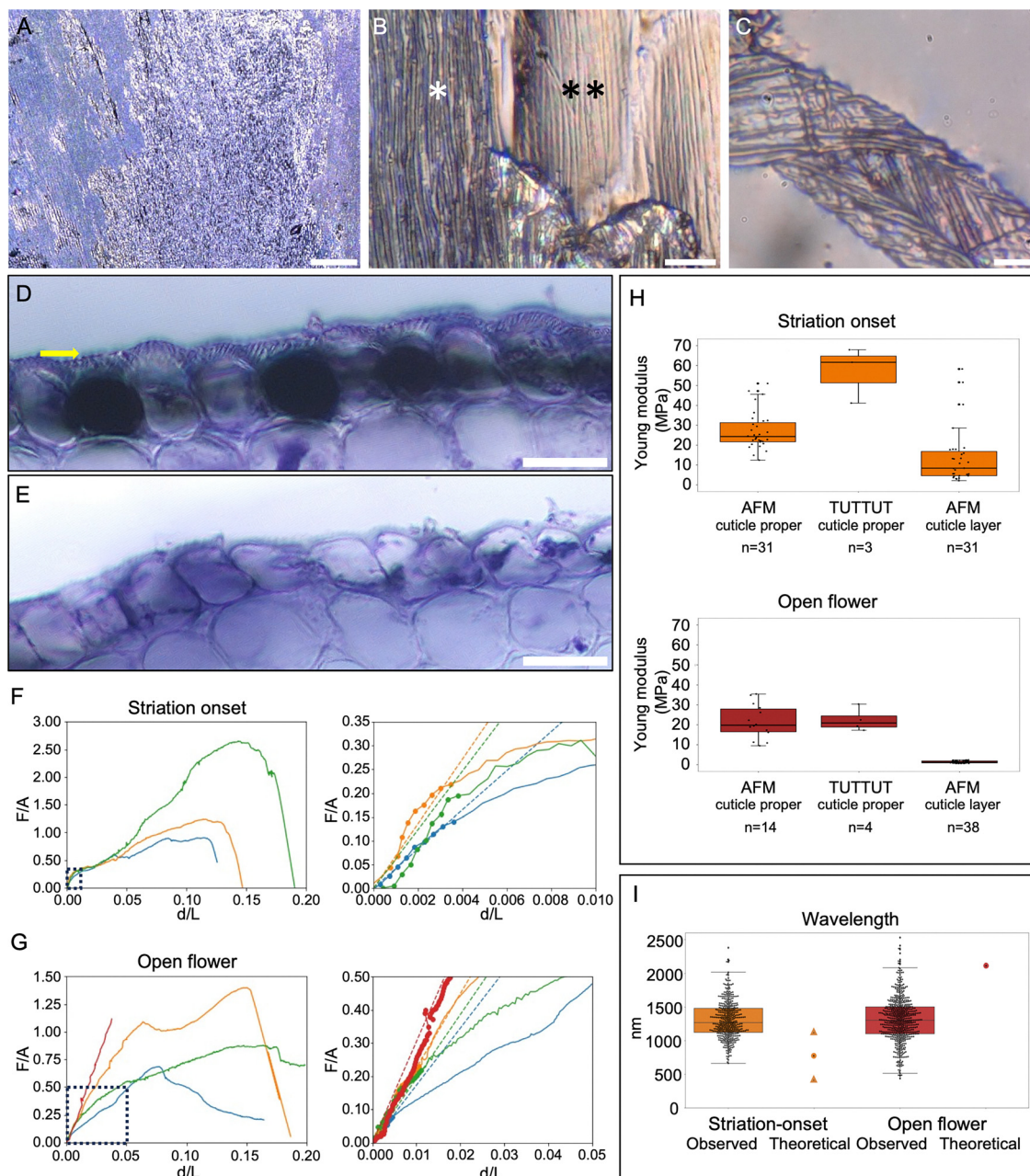


Fig. 3 Characterisation of stiffness of cuticular layers during striation in *Hibiscus trionum* petals. (A) and (B) Keyence images of a petal before and after cuticle proper removal. (B) Overview of a petal region after cuticle proper removal, cells still with the cuticle proper are indicated with *, cells without the cuticle proper are marked with **. (C) Isolated cuticle proper after being removed from the petal. (D) Cross section of an intact wild type petal (purple region, open flower), and (E) a petal after cuticle proper removal. Yellow arrow indicates the cuticle proper. (F) and (G) Stress vs strain values from the uniaxial tensile tester for ultrathin films (TUTTUT) measurements of the cuticle proper at the striation onset (F) and at the open flower stage (G). The left subplots show the full strain range responses and the right subplots are the zoom-in at the small strain regime indicated with dotted line boxes. The three samples of the onset stage have the moduli of 41.2, 67.9, and 61.6 MPa respectively. The four mature stage samples have the moduli of 17.3, 22.5, 19.3, and 30.4 MPa respectively. The fitting regime for linear elasticity is marked as dots, and the dashed lines are the linear fittings. For striation onset specimens, the fitting was based on the first 0.4% of average d/L values. For open flower specimens, the fitting was based on the first 1% of average d/L values. (H) Young's moduli values calculated from atomic force microscopy (AFM) and the TUTTUT measurements during striation onset and at the open flower stage. The box delimits the interquartile range (IQ3 minus IQ1), the horizontal line depicts the median, and the whiskers indicate the maximum ($Q3 + 1.5(IQR)$) and the minimum ($Q1 - 1.5(IQR)$); there are some outliers. (I) Observed vs theoretical wavelength of striations. Wavelength theoretical values were calculated using our measurements and eqn 1, values for observed wavelength in Hibiscus petals were reported in ref. 4, the circle indicates the predicted value using the average values for film thickness and Young's modulus of the film and substrate, the triangles indicate the predicted values considering the standard deviation for film thickness, and Young's modulus values. Scale bars: (A) 100 μm , (B) 10 μm , (C) 5 μm , (D) and (E) 20 μm .

materials increases dramatically: at the onset of striation initiation, it is around 2 and at the open flower stage it has increased to approximately 16 (based on AFM data). Future analyses will focus on the consequences of changing the timing of cuticle formation and cuticle stiffness changes during petal development.

The stiffness of cell layers can influence plant development and affect the shape and structure of an organ. Some changes in tissue mechanical properties (including stiffness) have previously been shown to occur during plant development, affecting cell division and expansion. In *Arabidopsis* roots, differential cell wall stiffness is associated with the elongation or differentiation zones.²⁷ In *Arabidopsis* fruits, the stiffness of the outer layer in the valves is constant during fruit elongation, however, a mutant affected in the degradation of cell wall components displayed an increase in fruit stiffness, affecting fruit elongation.²⁸ In the *Hibiscus* petals, we observed a decrease in stiffness of both the cuticle proper and the cuticular layer over time. With respect to the cuticle proper, this phenomenon could be related to exposure of the petal surface to the environment: the flower bud is closed during development, then opens and is exposed to the air with differences in humidity and temperature that might influence cuticle chemistry and mechanical properties. For the cuticular layer, we hypothesise that the even greater degree of softening that we recorded might reflect mechanical changes to the cell wall as rapid cell expansion occurs during petal growth. Cell expansion is dependent on expansin-induced loosening of bonds between cellulose and hemicellulose molecules,²⁹ which might be expected to reduce the stiffness of a material. Alternatively, given that the *Hibiscus* flower is open for a relatively short time (approximately 2 hours), it is possible that the softening we record in both layers represents the onset of senescence, involving changes in cell metabolism and turgor.

Previous studies of plant surface stiffness have largely treated the cuticle and cell wall as a single material, rather than the layers examined here. However, previous studies have explored the elastic moduli of isolated cuticles from tomato fruits, including during the growth of the tomato.^{30,31} The tomato fruit cuticle is extremely thick (ranging from 5.8 to 15.7 μm)³² and provides protection from water loss and pathogen infection, thereby allowing fruits to be transported and sold for significant time windows post-harvest. The cuticle proper in our Hibiscus petals is soft (23 MPa and 21.57 MPa for TUTTUT and AFM respectively, open flower) compared to published values for tomato fruit (ranging from 60 to 360 MPa).³² This big difference in Young's modulus between cuticles in petals and fruits could be related to the different functions of cuticle in different organs.^{17,32} In fruit the cuticle acts as a barrier to protect against dehydration, pathogens, and insect pests over a much longer time than the lifespan of a petal.³³

Although we observed changes occurring at the whole petal level during development, such as increases in cell size and layer thickness, we note that the striation patterns appear to be stable once they have formed. Lugo *et al.*⁴ also previously

showed that the striation wavelength is set when the pattern emerges and remains constant throughout petal growth. This suggests that upon striation formation, the deformation of the tissue seems to be locked despite the changes in cell size we observe from striation onset to flower opening. We hypothesise that this stability of the wrinkling wavelength across petal development could be an effect of the inelastic nature of biological materials during growth. This hypothesis is consistent with our observations after the isolation of the cuticle proper (mechanically or with enzymatic treatment), where the wrinkling patterns were still preserved in both the isolated cuticle proper and in the cuticular layer below (Fig. 3A–C). The onset of inelastic mechanical responses at larger strains is also consistent with the measured TUTTUT data (Fig. 3F and G) that show nonlinear responses at larger displacements.

When our data collected using AFM are applied to the theoretical model of Lugo *et al.*⁴ we find that the predicted value for the wavelength of the striations is consistent with the range of actual wavelength values measured on the petals, but not consistent with their mean or median. The living petals have striations with a mean wavelength of approximately 1300 nm, while our predictions using the data obtained here would be for a wavelength of around 800 nm at striation onset, increasing to around 2000 nm as the flower matures. Our hypothesis is that this discrepancy is generated by changing properties of this living system during development. During the striation onset stage, large parts of the flower are not yet producing striations. Given the very small surface measured with AFM, we could well be measuring the properties of parts of the tissue that are not yet capable of undergoing wrinkling. This is also suggested by a bigger spread of the AFM data and a bigger discrepancy between the AFM and TUTTUT measurements in the cuticle proper at the striation onset stage compared with the cuticle proper of the open flower. Additionally, the wavelength prediction using eqn (1) only applies to a flat domain *i.e.*, the tissue at the striation onset stage. At the later stages, when the cell surface and the petal surface are more curved, we observed bigger differences between the theoretical *vs.* the observed values. The simulations performed by Lugo *et al.*⁴ have shown how curvature can delay wrinkling onset and therefore could result in a longer wavelength than the one predicted by the simple equation. The stiffness values of the cuticular layer at the mature bud stage measured with AFM are greater than the values for the open flower (Table 1). At the same time the cuticle thickness has increased relative to the onset stage, and the cuticle stiffness has decreased only a little relative to the onset-stage, therefore the theoretical prediction of the wavelength increases. This suggests that during the night that divides the final two growth stages and sees the flower open fully in the morning (Fig. 1C–E both the growth of the layers and the softening of the substrate may result in the right material properties to develop the wavelength observed in the flowers. The dynamic nature of this system and the presence of a curved surface may explain the range of wavelengths observed in the flower. It is ultimately this imperfection in the pattern that creates the blue halo that increases salience to pollinators.²

Conclusions and perspectives

Here, we report the measurement of stiffness of cuticular layers through petal development. According to our analyses, the AFM and TUTTUT measurements overlap, indicating that both methods could be used to measure stiffness of cuticular layers in *Hibiscus* petals and in other living bilayer systems. Since AFM is more accessible for biological labs, we propose that our AFM protocol, taking into account depth of indentation, is sufficient to provide reliable data on stiffness of layered living systems. TUTTUT, a newly developing method, could provide future insight into larger strain mechanical responses, including the onset of inelastic behaviour.

The characterisation of *Hibiscus trionum* petals using multiple approaches has allowed us to describe the cuticle bilayer in the flower and the buckling phenomenon under the effect of compressive forces. We show that the equation used to describe materials reliably describes our biological system and we can predict the wavelength after buckling to a reasonable degree of accuracy by knowing the thickness of the film and the stiffness of the two layers. *Hibiscus trionum* petals, despite being a living and changing biological system, behave and buckle like other materials.

Our study also reveals the dynamic development of material properties, with tissue stiffness changing through developmental time. This dynamic property may allow multi-functionality that is difficult to achieve through a static material and may be of interest in the bioinspired design of new materials.

We aim to use the described protocol to dissect the contribution of genetic, biochemical, and environmental factors in the formation of softer or stiffer cuticular layers and their influence in the formation of cuticular patterns. Additionally, further analysis will be conducted to assess the contribution of other cellular features, such as cell curvature and cell shape, to the formation of the observed wrinkling patterns.

Author contributions

Conceptualisation: CAA, CC, AJC, BJG; methodology: CAA, CC, HF, AJC, BJG; investigation: CAA, CC, HF; validation: CAA, CC, HF, CAL, UP; formal analysis: CAA, CC, HF, CAL; resources: AJC, BJG; writing – original draft: CAA, HHU; writing – review & editing: HHU, AJC, BJG; visualisation: CAA, CC, HHU, HF, UP, CAL; supervision: CAA, CC; project administration: AJC, BJG; funding acquisition: AJC, BJG.

Conflicts of interest

The authors declare no competing interests.

Data availability

The raw data that support the findings of this study are available from the corresponding author, Beverley J. Glover, upon request.



The data supporting this article have been included as part of the supplementary information (SI). Supplementary information: Overview of the tip used in the atomic force microscopy experiments; atomic force microscopy maps; examples of force-displacement curves obtained in the AFM experiments; examples of data processing in the AFM experiments; finite element calibration of TUTTUT experiment; *Hibiscus trionum* petal mass increase over time; comparison of Young's modulus values in the cuticle proper. See DOI: <https://doi.org/10.1039/d4sm01406e>.

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