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REVIEW ARTICLE

Imaging the living plant cell: from probes to quantification

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Short title: New methods in live cell imaging

One sentence summary: Specific examples are used to illustrate some of the challenges of live cell imaging, from designing genetically encoded probes to choosing a pipeline for image analysis and quantification.

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Abstract

At the center of cell biology is our ability to image the cell and its various components, either in isolation or within an organism. Given its importance, biological imaging has emerged as a field of its own, which is inherently highly interdisciplinary. Indeed, biologists rely on physicists and engineers to build new microscopes and imaging techniques, chemists to develop better imaging probes, and mathematicians and computer scientists for image analysis and quantification. Live imaging collectively involves all the techniques aimed at imaging live samples. It is a rapidly evolving field, with countless new techniques, probes, and dyes being continuously developed. Some of these new methods or reagents are readily amenable to image plant samples, while others are not and require specific modifications for the plant field. Here, we review some recent advances in live imaging of plant cells. In particular, we discuss the solutions that plant biologists use to live image membrane-bound organelles, cytoskeleton components, hormones, and the mechanical properties of cells or tissues. We not only consider the imaging techniques per se, but also how the construction of new fluorescent probes and analysis pipelines are driving the field of plant cell biology.

1 **Introduction**

2 As recently described by Marc Somssich in his “short history of plant light microscopy”, the
3 invention of the microscope and its use to observe plant tissues “opened up a completely new
4 world previously hidden to the human eye” (Somssich, 2021). It notably led to the cell theory,
5 which proposed that the cell is the fundamental unit of life and placed the cell at the center of
6 organismal biology (Kierzkowski and Routier-Kierzkowska, 2019). In this review, we focus on
7 the recent advances made in the field of live imaging of plant cells.

8
9 From the point of view of probes, live imaging of plants, as in the rest of biology, was really
10 boosted by the discovery and use of fluorescent proteins (Chalfie, 2009; Somssich, 2021).
11 While new, improved fluorescent proteins in different colors are continuously being developed
12 (Lambert, 2019), most of the recent advances came from the development of genetically
13 encoded biosensors and reporters (Grossmann et al., 2018; Walia et al., 2018); we will describe
14 some of these advances here. On the microscopy side, confocal microscopy is the most widely
15 used method by far. Briefly, this technique relies on one or several pinholes that block out-of-
16 focus light and thus increase the contrast and resolution of fluorescent imaging by collecting
17 only (or mostly) the light coming out of the focal plane (**Table 1**) (Bayguinov et al., 2018).
18 Confocal microscopy is particularly well suited for imaging moderately thick and rather
19 transparent samples, such as a variety of plant tissues or organs. We will also introduce some
20 of the new imaging techniques that have increased the speed of acquisition, its sensitivity,
21 spatial resolution, or depth of acquisition (**Table 1**) (Grossmann et al., 2018; Clark et al., 2020).

22
23 There are already a number of excellent reviews that discuss live imaging in plants (see for
24 example (Sappl and Heisler, 2013; Berthet and Maizel, 2016; Grossmann et al., 2018; Komis
25 et al., 2018; Clark et al., 2020)). Here, rather than having a mostly technical and technological
26 focus, we decided to consider some of the classical problems in cell biology to illustrate 1) how
27 plant biologists use live imaging to address them, 2) what are the challenges in setting up live
28 imaging experiments, and 3) what are the solutions to overcome these pitfalls. To this end, we
29 will review some of the methods used to image the cytoskeleton, the plant endomembrane
30 network, and plant hormones and their activity. Finally, we will introduce an array of imaging
31 techniques that are being developed to study the biophysical and mechanical properties of plant
32 cells and tissues.

33 Visualization and quantification of the plant cytoskeleton

34 Markers for live imaging of the cytoskeleton

35 Actin and microtubule filaments are among the most fascinating structures in the cell. They are
36 highly dynamic and under constant remodeling, which quickly prompted the development of
37 live reporters to capture these ever-changing structures. In plants, one of the more reliable actin
38 reporters and one of the first to be described is the *Arabidopsis thaliana* Fimbrin-like, AtFim1
39 (**Table 2**) (McCurdy and Kim, 1998; Kovar et al., 2001; Voigt et al., 2005). The C-terminal
40 half of AtFim1 (aa 325–687; coined AtFim1 ACTIN-BINDING DOMAIN2 – fABD2) fused
41 to a fluorescent protein is more efficient at labeling the actin filaments than the full-length
42 protein and is therefore generally used as a standard for actin filament visualization *in vivo*
43 (Ketelaar et al., 2004; Sheahan et al., 2004; Wang et al., 2004). While the use of the mouse
44 Talin as a reporter has rapidly diminished due to side effects, the fABD2 domain has been
45 largely used to visualize the actin cytoskeleton (Wang et al., 2004). Yet, the strong expression
46 of the *GFP-fABD2-GFP* reporter has inhibitory effects on cell and organ growth; therefore, it
47 is crucial to use promoters with low or moderate expression levels (Wang et al., 2008; Dyachok
48 et al., 2014). The other commonly used reporter for actin filaments is a 17-amino-acid peptide
49 named LifeAct, which appears to be a faithful biosensor without extensively disrupting the
50 dynamics of the actin filaments (Riedl et al., 2008). While LifeAct decorates actin filaments
51 with minimum perturbation of their dynamics, *LifeAct* expression also needs to be optimized to
52 reach an expression level lower than for *fADB2* to prevent the bundling of actin filaments (Era
53 et al., 2009; Dyachok et al., 2014). The dynamic reorganization of the actin cytoskeleton can
54 be assessed at super-resolution by photoactivation localization microscopy, with the LifeAct
55 reporter fused to a photoactivatable fluorescent protein (Durst et al., 2014).

56

57 Like for the actin cytoskeleton, visualization of microtubules *in vivo* is often based on a
58 fluorophore-conjugated microtubule-associated protein. As such, the microtubule-binding
59 domain of the human Microtubule Associated Protein-4 MAP4 (MBD) fused to GFP became a
60 typical reporter used to visualize microtubules *in vivo* (**Table 2**) (Marc et al., 1998). Other
61 constructs with plant microtubule-associated proteins are also available, such as the
62 Microtubules Associated Protein of 65 kDa-1, MAP65-2 or MAP65-4 (Fache et al., 2010;
63 Lucas et al., 2011; Creff et al., 2015; Boruc et al., 2017). In these cases, careful attention needs
64 to be taken in the interpretation of the results, since such proteins enhance microtubule
65 polymerization and promote their nucleation, bundling, and stabilization (Fache et al., 2010).
66 The level of expression of such reporters should therefore be tightly monitored, as

67 developmental defects such as dwarfism or organ twisting are observed when their expression
68 is too high (Holzinger et al., 2009). Another approach is to directly tag the tubulin monomer
69 itself (Ueda et al., 1999). Fusions of Tubulin Alpha 6 (TUA6), TUA5, and Beta 6 (TUB6)
70 subunits to various fluorescent tags are used to describe the organization and dynamics of
71 microtubules *in planta* (Ueda et al., 1999; Nakamura et al., 2004; Abe and Hashimoto, 2005;
72 Liu et al., 2016). However, depending on the experiments and expression levels, the fluorescent
73 signal may appear more cytoplasmic using TUA6/TUB6 than MBD-based reporters (Doumane
74 et al., 2021). This makes quantification trickier, especially for automatic detection of individual
75 microtubule bundles, but at the same time, TUA6/TUB6 markers induce fewer side effects and
76 developmental phenotypes than MBD-based reporters. Nonetheless, as discussed for previous
77 reporters, high expression of TUA6 or TUB6 may still induce phenotypes, for example on cell
78 wall synthesis (Abe and Hashimoto, 2005; Burk et al., 2006).

79 While the markers described above are used to visualize the entire microtubule, some reporters
80 target subdomains of the microtubule, such as Arabidopsis End-Binding Protein-1a
81 (35Spro:AtEB1a-GFP, Chan et al. 2003). This protein labels the plus-ends of microtubules and
82 is visualized as a comet-like structure corresponding to the tip of the growing microtubule
83 (Chan et al., 2003; Bisgrove et al., 2008; Galva et al., 2014; Wong and Hashimoto, 2017; Elliott
84 and Shaw, 2018; Molines et al., 2018; Molines et al., 2020). This tool is particularly useful to
85 address the rate of microtubule growth or the angle between branched microtubules in a given
86 tissue or condition (Chan et al., 2009; Montesinos et al., 2020).

87 Whenever possible, it is best to use multiple markers to interpret live imaging experiments
88 based on both actin and microtubule fluorescent reporters. It is also important to keep in mind
89 that cytoskeleton reporters might not label the entire population of microtubules or actin
90 filaments due to competition with endogenous cytoskeleton regulators (Sadot and Blancaflor,
91 2019). As such, accurate detection of the cytoskeleton network by immunolocalization should
92 also be considered as an alternative (Belcram et al., 2016; Tichá et al., 2020; Du et al., 2021),
93 although it is not compatible with live imaging. In the animal field, vital fluorescent dyes that
94 can be added to the culture medium that label either actin or microtubules, such as SiR-actin or
95 Sir-tubulin, are becoming popular due to their ease of use (i.e. no need to genetically express a
96 reporter) (Lukinavičius et al., 2014; Melak et al., 2017). To our knowledge, these dyes have not
97 been extensively used in plant systems, very likely because they do not enter the cells, perhaps
98 due to the presence of the cell wall. In any case, like for genetically encoded markers, these
99 chemical probes also tend to affect cytoskeleton dynamics (Melak et al., 2017). Other technical

100 challenges are still blocking progress in the field, in particular the loss of the fluorescent signal
101 intensity in the inner tissues. The development of fluorescent markers expressed under the
102 control of tissue-specific promoters might help in this matter, as was done in the study of lateral
103 root initiation (Barro et al., 2019). Alternatively, the use of two-photon microscopy might help
104 to penetrate deeper into thick tissues (**Table 1**) (Grossmann et al., 2018; Mizuta, 2021).

105

106 **Model systems for live imaging of the cytoskeleton**

107 The cytoskeleton is very important for cell differentiation, elongation, and polarity. While live
108 imaging of cytoskeleton components has been carried out in many different cell types, it is
109 worth mentioning the few model systems that have been recurrently used over the years by
110 different groups. For example, root hairs and pollen tubes have extensively been used to study
111 cytoskeleton dynamics in tip growing cells (Ketelaar, 2013; Scholz et al., 2020; Xu and Huang,
112 2020). The tobacco (*Nicotiana tabacum*) pollen tube is, in particular, an excellent model for
113 live imaging studies of tip growth because they are big cells that are easy to transform and to
114 image (Kost et al., 1998; Klahre and Kost, 2006; Scholz et al., 2020; Xu and Huang, 2020;
115 Fratini et al., 2021). Microtubules are critical for anisotropic growth, which has been
116 extensively studied in the hypocotyl (Shaw, 2013; Lenarcic et al., 2017). The cytoskeleton is
117 also important for cell wall differentiation, which has been studied using a variety of systems,
118 including transient expression in *N. benthamiana* leaves and in differentiating xylem (Oda and
119 Fukuda, 2012; Oda, 2015). Of note, a cellular system was recently established to study long-
120 term microtubule rearrangements occurring during proto-xylem development (Schneider et al.,
121 2021). This system, based on xylem *trans*-differentiation upon induction of the transcription
122 factor VASCULAR-RELATED NAC DOMAIN7 (VND7), allows microtubule dynamics to be
123 followed at high temporal resolution and over the course of several hours.

124

125 The cytoskeleton is also extremely dynamic and essential during cell division. Historically, live
126 imaging of cell division has been performed using cell cultures such as tobacco BY-2 cells
127 (Buschmann, 2016). The maize (*Zea mays*) leaf is another system used to study cytoskeleton
128 dynamics during cell division (Rasmussen, 2016; Martinez et al., 2017). In Arabidopsis, the
129 shoot apical meristem has been used to study the link between cell division orientation,
130 microtubule dynamics, and mechanical forces (Louveaux and Hamant, 2013; Louveaux et al.,
131 2016). The shoot apical meristem is indeed an excellent model system for many live imaging
132 approaches, including cytoskeleton visualization and the study of cell division (Grandjean et
133 al., 2004; Heisler and Ohno, 2014; Tobin and Meyerowitz, 2016; Willis et al., 2016; Hamant et

134 al., 2019a). This is because 1) it develops relatively slowly and thus does not require fast
135 imaging systems, 2) its morphogenesis is mainly driven by events happening in the epidermis
136 (L1 layer) (Kutschera and Niklas, 2007; Savaldi-Goldstein et al., 2007; Vernoux et al., 2021),
137 which is easily amenable to light microscopy approaches and can be targeted by drugs or
138 exogenous hormonal treatments (Grandjean et al., 2004; Echevin et al., 2019; Brunoud et al.,
139 2020), and 3) it can be excised from the plant and grown *in vitro* for a few days (Grandjean et
140 al., 2004; Brunoud et al., 2020).

141

142 The root, particularly the root tip, is generally considered a model of choice by plant cell
143 biologists. This is because the root tip is thin and transparent (without the autofluorescence of
144 the chloroplasts), with cells that are not yet fully differentiated (and thus have small vacuoles,
145 an expanded cytoplasm, and a thin cell wall with reduced autofluorescence) and relatively slow
146 cytoplasmic streaming. However, this model still has some limitations. First, the root tip very
147 quickly grows out of the field of view (in roughly 30 minutes), which limits long time-lapse
148 approaches, for example to study cell division. This problem can now be solved using
149 unsupervised approaches to track root growth (Doumane et al., 2017; von Wangenheim et al.,
150 2017). For example, using genetically encoded actin reporters and automatic root tracking, actin
151 dynamics was recently imaged and quantified during plant cell division at unprecedented time
152 scales (Lebecq et al., 2021). Secondly, roots constantly reorient their growth according to the
153 gravity vector (Armengot et al., 2016), a response that is blocked when slides are mounted
154 horizontally.

155

156 **Quantification of cytoskeleton dynamics in live imaging experiments**

157 With recent advances in live-cell imaging, huge amounts of data are now generated for each
158 experiment. Post-acquisition processing and quantitative analysis of the dynamics and
159 organization of the cytoskeleton are the most time-consuming parts of the experimental
160 procedure. Indeed, quantitative information is now becoming the standard to study the
161 architecture and dynamics of the cytoskeleton (Autran et al., 2021). Quantification of
162 cytoskeleton dynamics is generally obtained through the analysis of time sequences obtained
163 either on single images or projected z-stacks. Using color-coded image sequence in the widely
164 used image analysis software ImageJ (Schindelin et al., 2012; Schneider et al., 2012), the shift
165 in the positions of bundles in interphasic cells can be visualized (Kuběňová et al., 2021). This
166 post-acquisition analysis can be coupled with the generation of a kymograph, depicting straight
167 lines when the cytoskeleton is immobile and wavy lines in the case of active movements

168 (Lindeboom et al., 2013; Doumane et al., 2021; Schneider et al., 2021). The degree of bundling
169 of the cytoskeleton in normalized image stacks can be obtained in a semi-automated way, using
170 a plot profile generated from the Gel Analyser ImageJ function (Molines et al., 2018). This
171 simple method allows one to rapidly compare the degree of bundling under different conditions
172 or in genetic backgrounds expressing the same fluorescent reporter. Further parameters can be
173 extracted from time series, such as the growth and shrinkage speed or the catastrophe and rescue
174 rates (Lindeboom et al., 2013; Schneider et al., 2019). Importantly, at the subcellular level, *in*
175 *vivo* imaging and quantification of the cytoskeleton in three dimensions is still challenging.
176 Collaborative projects between cell biologists and mathematicians with expertise in image
177 analysis might go a long way towards filling this gap.

178 One of the standards for the quantitative measurement of cytoskeleton organization and thereby
179 cell growth anisotropy is the ImageJ plugin FibrilTool (Boudaoud et al., 2014). This computing
180 method assesses the pixel intensity level in a region of interest (ROI) and generates a vector
181 tangent to the fibrils, giving us access to the anisotropy of the network in a semi-automatic
182 manner (Boudaoud et al., 2014) (see **Figure 1A** for an example). Such an approach has been
183 successfully used to study the anisotropy of the microtubule network after genetic perturbation
184 or pharmacological treatment in different systems (Robinson and Kuhlemeier, 2018; Riglet et
185 al., 2020; Zhao et al., 2020). Similar approaches were recently used to quantify how geometry
186 affects cytoskeletal organization by confining single cells (or protoplasts) within
187 microfabricated microwells of various geometries (see (Colin et al., 2020; Durand-Smet et al.,
188 2020) and the last paragraph of this review). This plugin has been integrated into the
189 MorphographX platform (de Reuille et al., 2015), thus allowing microtubule organization on
190 computer-assisted cell segmentations to be analyzed (see next paragraph).

191

192 **Live imaging of membrane lipids and organelles**

193

194 **Imaging the plasma membrane, a key to segmenting cells in tissues**

195 The ability to segment cells is crucial for morphodynamic approaches, and having good markers
196 specific to the cell contour is a pre-requisite for automatic segmentations (Hong et al., 2018). It
197 is possible to segment cells by labeling the cell wall. In particular, propidium iodide (PI) is a
198 red fluorescent dye that labels pectins in the cell wall and is often used in live imaging
199 approaches to label cell contour (Kierzkowski et al., 2019; Sede et al., 2020); however, it is
200 toxic to cells and affect growth, thus limiting long-term live cell imaging. Alternatively,

201 membrane dyes or fluorescently tagged plasma membrane proteins are often used to segment
202 cells when performing live imaging of growing tissues. A popular dye used to label the plasma
203 membrane is FM4-64. This dye can be directly applied to live cells or tissues because it
204 fluoresces only in a lipidic environment (Grandjean et al., 2004; Rigal et al., 2015; Doumane
205 et al., 2017). An important property of FM4-64 is that it cannot pass through membrane. Thus,
206 when applied to the imaging medium, it first labels the plasma membrane before labeling
207 internal compartments following endocytosis. FM4-64 and PI are convenient because they
208 fluoresce in red, which is compatible with green/yellow fluorescent reporters. However, both
209 FM4-64 and PI have a number of limitations. First, they strongly label external cell/tissue layers
210 but provide little or no labeling of internal layers. For example, in the root, the Casparian strip
211 forms an impermeable barrier, which restrict the diffusion of FM4-64 and PI in internal tissues
212 (i.e. the stele) (Alassimone et al., 2010). Secondly, they wash away and bleach over time, which
213 is problematic when performing long time-lapse acquisitions. In this case, they must be
214 regularly reapplied to the mounting medium, which is not always convenient and can lead to
215 variation in labeling intensities (Doumane et al., 2017). Thirdly, FM4-64 becomes internalized
216 through endocytosis overtime. This is actually a property of this dye that is often used to study
217 endocytic processes (Rigal et al., 2015). However, strong labeling of intracellular compartments
218 can be problematic for the automatic segmentation of cells.

219

220 As an alternative to FM4-64 labeling, transgenic lines stably expressing fluorescently tagged
221 plasma membrane proteins can be used. One of the most widely used proteins is LOW
222 TEMPERATURE INDUCED PROTEIN 6B (Lti6b, also called RARE-COLD-INDUCIBLE
223 2B/RCI2b/At3g05890) and its tandem duplicated gene Lti6a/RCI2A (At3g05880) (**Figure 1B**)
224 (Kim et al., 2021). These two proteins were initially identified by Sean Cutler and colleagues
225 in a screen for GFP-tagged proteins with interesting localizations. The corresponding transgenic
226 lines are sometimes referred to as 29-1 and 37-26, which are the numbers of the original
227 transgenic lines identified in this screen (**Table 3**) (Cutler et al., 2000). Red and yellow variants
228 are now available as well, increasing the palette of available transgenic lines (Elsayad et al.,
229 2016; Noack et al., 2021). Other proteins that are often used as plasma membrane markers
230 include aquaporins such as PIP2;1/PIP2a (also initially identified in Cutler et al. as line Q8) or
231 PIP1;4 (Cutler et al., 2000; von Wangenheim et al., 2016), the formin FH6 (De Rybel et al.,
232 2010), syntaxins such as SYP122 or NPSN12 (Assaad et al., 2004; Geldner et al., 2009;
233 Vermeer et al., 2014; Barberon et al., 2016), lipid anchored fluorescent proteins (e.g.
234 myristoylation, acylation, prenylation (Vermeer et al., 2004; Simon et al., 2016; Willis et al.,

235 2016; Yang et al., 2021), or lipid binding proteins (Simon et al., 2014; Simon et al., 2016)
236 (**Table 3**). Genetically encoded fluorescent plasma membrane markers avoid some but not all
237 of the above-mentioned drawbacks of FM4-64. For example, it is not always easy to obtain a
238 strict plasma membrane localization. Indeed, transmembrane proteins traffic through the
239 endomembrane system to reach the plasma membrane and are degraded in the vacuole. This
240 can be problematic for pH resistant fluorescent proteins (e.g. mCHERRY, mCITRINE) that are
241 sometimes prominently seen in the vacuoles in some cell types or under certain growth
242 conditions (e.g. lower pH of the vacuole in the dark). Extrinsic proteins may partition between
243 the plasma membrane and the cytosol, which can affect segmentation. Other drawbacks of such
244 reporter lines include i) the bleaching of fluorescent proteins when imaged at high frequency
245 rates, ii) the requirement for transgenesis, which may not be possible when studying certain
246 species, and iii) the need to cross into the desired genetic background prior to imaging, which
247 is time consuming.

248

249 Once the plasma membrane (or alternatively the cell wall) is labeled with sufficient contrast,
250 several software programs/algorithms have been developed to allow automatic extraction of
251 cell contours, plant cell segmentation, and lineage tracing, including MorphographX,
252 MARS/ALT, PlantSeg, and SurfCut (Fernandez et al., 2010; de Reuille et al., 2015; Erguvan et
253 al., 2019; Strauss et al., 2019; Wolny et al., 2020) (See **Figure 1B** for an example of
254 segmentation using MorphographX). Importantly, the plasma membrane is not a uniform
255 compartment but is instead made up of a mosaic of small domains that are referred to as
256 microdomains ($>1 \mu\text{m}$) or nanodomains ($<1 \mu\text{m}$) (Ott, 2017; Jaillais and Ott, 2020).
257 Microdomains include polar domains within plant cells (see (Ramalho et al., 2021) for a
258 comprehensive review on the topic) as well as plant-microbial interfaces (Ott, 2017).
259 Nanodomains are by definition small, and often their size is below the diffraction limit of optical
260 microscopy. Several techniques have been used to visualize nanodomains in the living plant
261 plasma membrane and to probe their dynamics, notably Total Internal Resonance Fluorescence
262 Microscopy (TIRFM), PhotoActivated Localization Microscopy (PALM), and Single Particle
263 Tracking (SPT) techniques (**Table 1**) (Martiniere et al., 2012; Hosy et al., 2015; Gronnier et
264 al., 2017; Wang et al., 2018; Martiniere et al., 2019; Platre et al., 2019; Zhang et al., 2019;
265 Smokvarska et al., 2020; Bayle et al., 2021; Noack et al., 2021). These methods have revealed
266 a number of nanodomain-resident proteins, such as Remorins, Flotilins, HYPERSENSITIVE
267 INDUCED REACTION proteins (HIRs), and receptor-like kinases (Li et al., 2012; Bucherl et
268 al., 2017; Daněk et al., 2020; Gronnier et al., 2020; Jaillais and Ott, 2020; Gouguet et al., 2021;

269 Martinière and Zelazny, 2021), as well as some proteins with a dynamic association with
270 nanodomains, such as small GTPases from the RHO-OF-PLANTs (ROP) family (Platre et al.,
271 2019; Smokvarska et al., 2020; Bayle et al., 2021; Fuchs et al., 2021; Smokvarska et al., 2021).
272 Both microdomains and nanodomains not only have a specific protein composition but also
273 accumulate specific lipid species (see the section on lipids below) and are highly interconnected
274 with the rest of the endomembrane network via both the vesicular and non-vesicular transport
275 of materials.

276

277 **Imaging intracellular trafficking, fast and tiny!**

278 The plasma membrane is part of the endomembrane system, a network of membranes
279 interlinked by vesicular trafficking and direct membrane contacts (Boutté and Jaillais, 2020).
280 This system includes the endoplasmic reticulum and the connected nuclear envelope, the Golgi
281 apparatus and trans-Golgi Network (TGN), endosomes, vacuoles, and lysosomes, and the
282 plasma membrane (Boutté and Jaillais, 2020). A number of dyes label specific parts of the
283 endomembrane network. As mentioned above, FM4-64 is a prominent tool used to study the
284 dynamics of endocytic processes because it can be used in pulse-chase experiments (Rigal et
285 al., 2015; Johnson et al., 2020). Depending on the timing following FM4-64 treatment, it can
286 either label i) the plasma membrane specifically, ii) the plasma membrane and early
287 endosomes/TGN, or iii) the plasma membrane, early and late endosomes, and the tonoplast
288 (Dettmer et al., 2006; Jaillais et al., 2006; Jaillais et al., 2008; Geldner et al., 2009; Rigal et al.,
289 2015). There are also dyes that label the vacuole, such as BCECF [2',7'-Bis-(2-Carboxyethyl)-
290 5-(and-6)- Carboxyfluorescein] (Scheuring et al., 2016; Takemoto et al., 2018). Combined with
291 fluorescence recovery after photobleaching (FRAP, **Table 1**), BCECF allowed the connection
292 between vacuoles within cells to be studied (Scheuring et al., 2016).

293

294 For the most part, plant cell biologists use fluorescent fusions with proteins targeted to specific
295 compartments. The number of such fluorescent markers exploded since the publication of the
296 Cutler collection, which initially identified markers for many cellular compartments (Cutler et
297 al., 2000). In addition, a landmark resource in terms of endomembrane markers is the Waveline
298 collection, which not only provided multiple markers for each compartment, but did so in
299 several colors (Geldner et al., 2009). Having markers of different colors is critical for
300 colocalization experiments. Indeed, most intracellular compartments seen under a confocal
301 microscope look like dots and cannot be irrefutably identified based on their morphology alone.

302 The sensitivity to drugs can be used to discriminate between different membrane compartments
303 (Geldner et al., 2003; Dettmer et al., 2006; Jaillais et al., 2006; Jaillais et al., 2008; Worden et
304 al., 2014; Kania et al., 2018; Mishev et al., 2018), but colocalization is the gold standard.
305 Importantly, as discussed above for plasma membrane proteins, strict localization in a single
306 compartment is very rare. To obtain a robust idea of the localization of a given protein, it is thus
307 essential to perform quantitative colocalization with many different markers. Quantification of
308 colocalization can be tricky; several methods for doing this are described in some excellent
309 reviews (Bolte and Cordelieres, 2006; Lagache et al., 2015; Aaron et al., 2018; Lagache et al.,
310 2018).

311
312 There are two major difficulties when studying the dynamics of the endomembrane system.
313 First, vesicles and membrane domains are often tiny, being at or below the optical resolution of
314 light microscopy (~250 nm)(Sahl et al., 2017; Schermelleh et al., 2019). Second, membrane
315 trafficking is fast, with certain compartments moving tens of micrometers per minute, notably
316 due to cytoplasmic streaming (Luo et al., 2015). In term of resolution, there are more and more
317 examples of super-resolution microscopy methods used in plants (Komis et al., 2015b; Komis
318 et al., 2015a; Schubert, 2017; Shaw et al., 2019; Bayle et al., 2021). These methods can provide
319 large gains in resolution, such as PALM (Hosy et al., 2015; Gronnier et al., 2017; Martiniere et
320 al., 2019; Platre et al., 2019; Bayle et al., 2021) and Stimulated Emission Depletion Microscopy
321 (STED) (Kleine-Vehn et al., 2011; Demir et al., 2013) or provide ultrafast high resolution
322 imaging, such as super-resolution confocal live imaging microscopy (SCLIM) (**Table 1**)
323 (Naramoto et al., 2014; Uemura et al., 2014; Uemura et al., 2019; Shimizu et al., 2021). SCLIM
324 in particular appears to be well suited to study membrane trafficking events in plants. For
325 example, 3-colored 4D imaging of the Golgi and the TGN was recently reported in Arabidopsis
326 roots, allowing highly specialized subdomains within the TGN to be identified (Shimizu et al.,
327 2021).

328
329 To image events that occur at or close to the plasma membrane, the technique of choice is TIRF
330 microscopy (or derivatives of the TIRF technique such as variable angle epifluorescence
331 microscopy (VAEM — VA-TIRFM), which is a very sensitive technique because it does not
332 collect any out-of-focus light (**Table 1**) (Konopka et al., 2008; Konopka and Bednarek, 2008;
333 Gronnier et al., 2017; Johnson and Vert, 2017; Platre et al., 2019; Johnson et al., 2020;
334 Narasimhan et al., 2020; Smokvarska et al., 2020; Bayle et al., 2021). TIRF microscopy has
335 mainly been used to study endocytosis, but also cellulose synthesis and cytoskeleton dynamics,

336 and it can be combined with structured illumination microscopy (SIM) to achieve fast super-
337 resolved acquisition (**Table 1**) (Johnson et al., 2021). Quantification methods to study
338 endocytosis in plants were recently reviewed (Dragwidge and Van Damme, 2020; Johnson et
339 al., 2020).

340

341 **Imaging lipids**

342 Unlike proteins, membrane lipids cannot be genetically tagged with a fluorescent protein. While
343 it is possible to use cellular fractionation or immunolocalization, these techniques are not
344 amenable to live samples. By contrast, genetically encoded biosensors are compatible with live
345 imaging (Platre and Jaillais, 2016). In their simplest form, lipid biosensors are sometimes
346 referred to as translocation sensors (Platre and Jaillais, 2016; Wills et al., 2018). They consist
347 of an isolated lipid binding domain known to interact stereo-specifically with a given lipid
348 species fused with a fluorescent protein (**Figure 2A**). These domains are generated in the
349 cytosol and are targeted to the membranes *via* interaction with their cognate lipids, hence the
350 term “translocation sensors”. These sensors were instrumental in studying the subcellular
351 accumulation of lipids and helped draw a map of lipid localization in plant cells (**Table 4**)
352 (Vincent et al., 2005; Vermeer et al., 2006; van Leeuwen et al., 2007; Vermeer et al., 2009;
353 Simon et al., 2014; Simon et al., 2016; Hirano et al., 2017; Noack and Jaillais, 2017; Vermeer
354 et al., 2017; Hirano et al., 2018; Platre et al., 2018; Noack and Jaillais, 2020; Xing et al., 2020;
355 Ito et al., 2021). However, like any genetically encoded biosensors, they have inherent caveats
356 including i) competition with endogenous lipid binding proteins, ii) potential masking of their
357 endogenous ligands, and iii) the fact that these lipid binding domains usually rely on additional
358 membrane features for localization (Heilmann, 2016; Platre and Jaillais, 2016; Dubois and
359 Jaillais, 2021). In addition, they mostly recognize the lipid head groups and, in fact, are
360 available only to study anionic phospholipids. Indeed, no biosensors for abundant structural
361 phospholipids, sterols, or sphingolipids have been characterized to date. This is mostly due to
362 the lack of known lipid binding domains with specific binding to these lipids.

363

364 Because these sensors are produced in the cytosol, they are designed to study the lipid
365 embedded in the cytosolic leaflets, not the extracellular or luminal membrane leaflet, which is
366 an additional limitation of these sensors (**Figure 2A**). Finally, because they are based on
367 translocation, which can be tricky to quantify, these sensors are useful for studying the
368 subcellular localization of anionic lipids, but are of limited interest for studying the amounts of
369 lipids in different cells or tissues (Colin and Jaillais, 2019). Quantification of the relative levels

370 of lipids can be achieved using fluorescence resonance energy transfer (FRET)-based lipid
371 sensors (**Figure 2B**) (Platre and Jaillais, 2016). To date, there is only one such sensor available
372 in plants for phosphatidic acid (PA) (Li et al., 2019b). This ratiometric sensor, named PAleon,
373 is based on a PA-binding domain, which is sandwiched between two fluorescent proteins (a
374 FRET donor and acceptor, **Table 1**) and constitutively anchored to the plasma membrane (Li
375 et al., 2019b; Mamode Cassim and Mongrand, 2019). PA-binding triggers a conformational
376 change in the sensor, which decreases the distance between the acceptor and donor fluorescent
377 proteins and thus a change in FRET (**Figure 2B**). Using PAleon, PA levels were shown to
378 rapidly change upon abiotic stress, which was known from previous biochemical studies. In
379 addition, these changes are highly tissue specific in the root, a feature that could not be
380 addressed using traditional biochemical approaches (Li et al., 2019b). FRET-based sensors for
381 other lipids have been used in animal cells (Platre and Jaillais, 2016; Wills et al., 2018) and are
382 eagerly awaited for studying and quantifying the levels of other lipids in plants. Other
383 approaches that could complement the biosensor approaches are based on in vivo lipid labeling,
384 for example via click-chemistry (Neef and Schultz, 2009; Tamura et al., 2020). These
385 approaches are starting to be available for plant samples (Paper et al., 2018), but as far as we
386 know, they have not yet been used on live plant tissues. It is also possible to use exogenous
387 treatments with fluorescently labeled lipids (Poulsen et al., 2015; Zhang et al., 2020b; Susila et
388 al., 2021), but it might be tricky to assess whether the localization of exogenously added lipids
389 reflects the true localization of endogenous lipids (in term of subcellular accumulation, leaflet
390 association, and potential degradation of the fluorescent lipid) (Grabski et al., 1993).

391

392

393 **Live imaging of plant hormones**

394

395 **Imaging the transcriptional output of hormones**

396 Recent years have seen an explosion in the number of genetically encoded biosensors, mainly
397 developed in Arabidopsis, to detect hormones at high spatio-temporal resolution within living
398 tissues using fluorescence microscopy (for recent reviews and a more exhaustive discussion on
399 genetically encoded biosensors see: (Walia et al., 2018; Martin-Arevalillo and Vernoux, 2019;
400 Isoda et al., 2021)). Here we highlight the most commonly used of these biosensors (**Table 5**).
401 A pioneering work that initiated these developments is the construction of the DR5 auxin
402 transcriptional sensor (Ulmasov et al., 1997; Sabatini et al., 1999; Ulmasov et al., 1999;
403 Benkova et al., 2003; Ottenschlager et al., 2003) and its more recent derivative DR5v2 (Liao et

404 al., 2015). Both consist of a synthetic auxin-responsive promoter, with multiple binding sites
405 for Auxin Response Factors (ARFs), driving the expression of a fluorescent protein (FP)
406 (**Figure 3A**). Indeed, plant hormones regulate gene expression via transcription factors specific
407 to each pathway that recognize specific binding sites (Larrieu and Vernoux, 2015). Strategies
408 similar to the one used for DR5 were then leveraged to design transcriptional biosensors for
409 cytokinins (Muller and Sheen, 2008; Zurcher et al., 2013; Steiner et al., 2020), ethylene
410 (Stepanova et al., 2007), and abscisic acid (ABA) (**Table 5**) (Wu et al., 2018). While
411 endogenous promoters of hormone-responsive genes have also been used to analyze the
412 transcriptional responses to hormones, the use of synthetic promoters increases the specificity
413 of the response of the biosensor to a given hormone. Transcriptional biosensors have provided
414 invaluable data on the physiology and roles of these four plant hormones in development (Isoda
415 et al., 2021). Importantly, hormone signaling pathways include feedback mechanisms and thus
416 have non-linear topologies. A classic example is the auxin pathway, which includes negative
417 feedback (Weijers and Wagner, 2016). Therefore, transcriptional biosensors of hormones do
418 not have an activity that is linearly dependent upon hormone levels. Instead, they provide
419 information on the processing properties of the signaling pathway that functions downstream
420 of the hormone. In addition, the activity of the transcription factors controlling the expression
421 of transcriptional biosensors might be regulated by other signals. Thus, transcriptional
422 biosensors can also be influenced by crosstalk between pathways (Nemhauser et al., 2004;
423 Jaillais and Chory, 2010).

424

425 **Measuring hormonal input**

426 Understanding how a given hormone regulates transcriptional responses within a tissue requires
427 direct information about the distribution of the hormone to be obtained. Two complementary
428 strategies have been used in parallel to tackle this challenge. The first strategy is based on the
429 observation that several hormones (auxin, jasmonates, gibberellins [GA], ABA, salicylic acid,
430 strigolactones, and karrikin) trigger rapid degradation of signaling effectors through
431 polyubiquitination by the Skp-Cullin-F-box (SCF) complex (Larrieu and Vernoux, 2015). This
432 has led to the design of degradation-based biosensors. This strategy was first implemented for
433 auxin with the development of the DII-VENUS biosensor (Brunoud et al., 2012) and its
434 ratiometric versions R2D2 and qDII (Liao et al., 2015; Galvan-Ampudia et al., 2020) (**Figure**
435 **3B** and **Table 5**). The level of the DII-VENUS synthetic protein is inversely correlated to the
436 concentration of auxin across a large range of concentrations, allowing auxin distribution to be
437 mapped at cellular resolution during development (For a specific review on this subject see

438 (Martin-Arevalillo and Vernoux, 2019)). This auxin degradation-based biosensor was shown
439 to function in Arabidopsis and in a variety of other plants such as maize (Mir et al., 2017),
440 Brachypodium (O'Connor et al., 2017), and more recently mosses (Landberg et al., 2021),
441 demonstrating the wide applicability of this design to evolutionarily distant plants. Synthetic
442 degradation-based biosensors have also been generated for jasmonates (Jas9-VENUS: (Larrieu
443 et al., 2015)), GA (qRGA^{mPFYR}: (Shi et al., 2021)), and strigolactones (Strigo-D2: (Song et al.,
444 2021)) using a ratiometric design. While degradation-based biosensors have proven to be
445 powerful and easy-to-use tools to analyze hormone contents in living tissues, they also have a
446 number of limitations. The detection remains indirect, as degradation of the biosensor uses the
447 hormone perception cellular machinery, which can induce detection biases, for example upon
448 differential expression of receptors (Vernoux et al., 2011; Brunoud et al., 2012). In addition,
449 their spatial definition is limited to the cellular scale or above, and they cannot detect rapid
450 variations in hormone levels, as they need to be re-synthesized following degradation.

451
452 The design of direct biosensors (i.e. biosensors that autonomously detect hormones) is a second
453 strategy that has been used in a handful of studies to detect hormone distribution even below
454 the cellular scale. FRET biosensors have been developed for ABA (ABACUS, ABAleon,
455 SNACS) (Jones et al., 2014; Waadt et al., 2014; Zhang et al., 2020a), GA (GPS1) (Rizza et al.,
456 2017), and more recently auxin (AuxSen) (Herud-Sikimic et al., 2021) (**Table 5**). FRET
457 biosensors use two FPs and the physical property of a donor FP excited at a certain wavelength
458 to transfer energy to an acceptor FP that will then fluoresce (**Table 1**). Here, this energy transfer
459 is modified by the binding of the hormone (**Figure 3C and D**). The FRET biosensors allow for
460 the rapid, quantitative detection of hormones within living tissues in Arabidopsis, where they
461 have been tested so far, and have been used to follow the hormone distribution dynamics during
462 developmental processes and environmental responses (Jones et al., 2014; Waadt et al., 2014;
463 Rizza et al., 2017; Waadt et al., 2020; Herud-Sikimic et al., 2021). While both the ABA and
464 auxin FRET biosensors have been shown to function in different intracellular compartments
465 (Jones et al., 2014; Herud-Sikimic et al., 2021), FRET biosensors are yet to be used to analyze
466 hormone distribution in different cell compartments or in the apoplast. This is notably, but
467 certainly not exclusively, a key missing piece of information for auxin given that multiple
468 intracellular transporters regulate auxin responses (Sauer and Kleine-Vehn, 2019). FRET
469 biosensors are not without limitations. Notably ABACUS, ABAleon, and GPS1 expression
470 leads to hypersensitivity to their hormone target. The FRET activity of GPS1 is also partly
471 reversible, and the range of concentrations detected by the existing FRET sensors might not

472 cover the entire range of endogenous concentrations (for an exhaustive comparison, see: (Isoda
473 et al., 2021)). Further optimization will certainly allow these limitations to be minimized
474 (Waadt et al., 2020) or even eliminated.

475

476 **Using quantitative live imaging to understand hormonal processing**

477 The different types of biosensors currently available provide a powerful toolbox to bring our
478 knowledge of hormone action during developmental and environmental responses to the next
479 level, from the cellular scale to the plant scale and even the population scale. Such technology
480 opens up extensive possibilities. For example, transcriptional and degradation-based/FRET
481 biosensors could be combined to understand how hormonal signals are dynamically processed
482 by signaling pathways to induce downstream changes in gene expression in living tissues. This
483 has been done for auxin by combining DR5 and DII-VENUS or qDII biosensors, revealing
484 differences in auxin sensitivity between functional domains of the shoot apical meristem and
485 the requirement for sustained exposure to high auxin levels for the induction of transcription
486 (Vernoux et al., 2011; Ma et al., 2019; Galvan-Ampudia et al., 2020). Biosensors for different
487 hormones could also be combined to understand their respective contributions to developmental
488 and environmental responses, such as for auxin and cytokinins, which often act antagonistically.
489 A large number of FRET sensors are also available for detecting endogenous metabolites and
490 small molecules (For review: (Walia et al., 2018; Isoda et al., 2021)), which could be combined
491 with hormone biosensors. This was recently done with ABA FRET biosensors and biosensors
492 for Ca²⁺, protons, chloride, H₂O₂, and glutathione redox potential (Walia et al., 2018; Waadt et
493 al., 2020). This study showcased how the effects of hormones on key secondary messengers
494 can be followed at high spatio-temporal resolution, demonstrating (for example) that GA does
495 not trigger rapid changes in pH or Ca²⁺. We expect that this toolbox will continue to be
496 developed in the near future. For example, sensors for strigolactones have been tested in
497 protoplasts (Samodelov et al., 2016; Chesterfield et al., 2020; Braguy et al., 2021) and are now
498 emerging *in planta* (Song et al., 2021). More such sensors will certainly emerge.

499

500 **Live imaging of the mechanical properties of cells and tissues and their** 501 **responses to forces**

502

503 In the last decades, biophysical approaches have been developed to probe the mechanical
504 properties of plant cells and their response to forces. Below, we review how live imaging has
505 taken on central importance in the emergence of this field of cell and developmental biology.

506

507 **Atomic force microscopy: probing for cell mechanical properties**

508 Atomic force microscopy (AFM) belongs to the family of scanning probe microscopy (SPM)
509 techniques, where a tip (or probe, usually with a nanometric radius) scans the surface of a
510 sample (Binnig et al., 1986). While in the case of optical or electron microscopies, topographic
511 information about the sample is gathered using the transmission or reflection of a beam, in the
512 case of AFM, it is the interaction force between the tip and the sample surface that is used. In
513 the case of contact mode operation, for example, the tip scans the surface while the system
514 monitors the tip-sample force and acts to maintain it at a constant level: if the sample surface is
515 not atomically flat and perfectly horizontal (i.e. lying on the xy scanner's plane), the tip has to
516 be moved up and down to maintain the force unchanged. Those displacements are then collected
517 to reconstruct a 3D topography of the surface. Depending on the tip used and the scanning
518 conditions, lateral and vertical resolutions may be <1 nm. Since this type of microscope can
519 easily be operated in liquid medium, its application in biology, particularly for living samples,
520 is rather natural and advantageous compared to other microscopy techniques.

521

522 Beyond topography, AFM allows any type of interaction forces to be detected, such as
523 electrostatic, van der Waals, or contact forces or specific interaction forces between the tip and
524 the sample, down to few piconewtons (pN). In addition, the tip can be used to apply forces at
525 the surface of a sample while measuring the resulting deformation (indentation) in order to
526 determine its mechanical properties (e.g., Young's modulus, viscoelastic properties).

527

528 Understanding the role of plant cell wall mechanics is essential for explaining the mechanisms
529 underlying developmental processes and morphogenesis (Hamant and Traas, 2010; Mirabet et
530 al., 2011; Sapala et al., 2018; Landrein and Ingram, 2019; Vernoux et al., 2021). Indeed, along
531 with genetic regulation and growth factors, the mechanical properties of the cell wall are tightly
532 regulated: for example, cell wall softening is required to allow cell growth. AFM allows these
533 properties to be measured and the way they change within/between organs, genotypes or
534 developmental stages to be studied (Milani et al., 2011; Peaucelle et al., 2011; Yakubov et al.,
535 2016). AFM can also be coupled with fluorescent microscopy to provide correlative
536 information between the mechanical properties of a cell/tissue and the expression of marker
537 genes (Milani et al., 2014). Elastic modulus maps can be generated by creating a series of force
538 curves (where the tip is alternatively placed onto and withdrawn from the surface) on a matrix
539 defined for a ROI in the sample (for advice on how to set up this type of experiment, see for

540 example (Bovio et al., 2019) and (Braybrook, 2015)). These curves are often analyzed using
541 standard contact models (e.g. Hertz, Sneddon, and so one), which can be used to calculate the
542 elastic modulus per curve. More advanced measurements can also be set up to study the
543 sample's viscoelastic properties (for examples in animal cells, see (Alcaraz et al., 2003) and
544 (Rother et al., 2014)) or to evaluate cell turgor pressure at the single cell (Beauzamy et al., 2015;
545 Long et al., 2020) or organismal level (Beauzamy et al., 2016; Creff et al., 2021).

546

547 Despite their versatility and high lateral resolution, scanning probe techniques are intrinsically
548 limited to the study of the sample's surface. More recently, new techniques known as optical
549 or photo-acoustic elastographies (Larin and Sampson, 2017; Singh and Thomas, 2019) have
550 been developed that provide information on the mechanical properties of the volume of a
551 biological sample. One of these techniques is Brillouin microscopy, which uses Brillouin
552 scattering to extract the longitudinal storage moduli of samples (Antonacci et al., 2020). This
553 technique is based on the inelastic scattering of an incident photon by a phonon (pressure wave)
554 of the sample. The process is similar to Raman scattering, but instead of modes of vibration of
555 single molecules, the information is retrieved from propagating phonons, thus providing access
556 to the mechanical properties of the material (Antonacci et al., 2020). This technique has already
557 been applied to plant tissues (Elsayad et al., 2016). However, it is still in its early stage of
558 development, meaning that the experimental set-up and analysis framework have to be
559 optimized to provide specific and reliable information on the mechanical properties of
560 biological samples.

561

562 **Measuring cellular responses to forces**

563 Plant organs are exposed to specific patterns of mechanical forces that can be perceived by cells
564 and influence key processes such as growth, division, polarity, and gene expression (Landrein
565 and Ingram, 2019). At the single cell level, mechanical stress builds up from the hydrostatic
566 pressure of the cell (i.e. turgor), which puts the surrounding walls under tension and induces
567 growth when the yielding threshold of these walls is exceeded (Lockhart, 1965). At the organ
568 level, mechanical stresses often build up from mechanical conflicts caused by differences in
569 mechanical properties (pressure and wall properties) between cells and tissues (Kutschera and
570 Niklas, 2007; Hamant et al., 2008). As stress patterns are of a purely physical nature, they can
571 be predicted using mechanical models (Hamant et al., 2008; Heisler et al., 2010; Bozorg et al.,
572 2014; Sampathkumar et al., 2014), but they cannot be directly measured easily. However, they
573 can also be indirectly assessed by measuring the turgor pressure of the cell, the strain (i.e.

574 deformation) they induce (notably at the membrane or in the cell wall), and the physiological
575 response they trigger in the cell.

576

577 Cell turgor can be directly measured in living tissues using a pressure probe, but this method is
578 invasive and difficult to use for small cells (Beauzamy et al., 2014). A less-invasive method has
579 thus been developed in which turgor pressure values are extracted based on indentations
580 generated with an atomic force microscope (Beauzamy et al., 2015). This technique was
581 recently used to unravel differences in pressure between cells in the epidermis of the shoot
582 apical meristem (Long et al., 2020). However, this method is indirect, as pressure information
583 must be extracted from force measurements using physical models, and this method cannot be
584 used to measure cell turgor in inner tissues. To overcome these limitations, a new FRET sensor
585 was recently developed to directly probe cell osmolarity (Cuevas-Velazquez et al., 2021). This
586 sensor is based on the use of an intrinsically disordered protein that is normally expressed under
587 water deficit conditions, and whose structure depends on the osmolarity of the medium.
588 Measuring the strain (elastic or plastic deformation) induced by mechanical forces on the cell,
589 and notably at the membrane or within the cell wall, is challenging. Microviscosity sensors
590 were recently developed to probe the mechanical environments of different cell compartments
591 (Michels et al., 2020). These sensors are molecular rotors whose rate of intramolecular rotation,
592 and thus their fluorescent lifetime (imaged by FLIM, **Table 1**), depends on their mechanical
593 environment. Microviscosity sensors have been used to unravel the existence of specific
594 patterns of membrane and wall microviscosity in roots and pavement cells. These patterns have
595 been linked to changes in membrane and wall composition but also to spatial and temporal
596 variations in membrane and wall tension, notably in response to changes in turgor pressure.
597 These microviscosity FLIM sensors thus appear to be unique tools for assessing mechanical
598 stress patterns within plant organs (Michels et al., 2020).

599

600 Mechanical forces could also be visualized and measured based on the response they induce in
601 the cell. The mechanisms through which cells are able to sense forces in plants are largely
602 unknown. It has been hypothesized that mechanical stress could be perceived at the interface
603 between the plasma membrane and the cell wall through receptor-like kinases (such as
604 FERONIA) and/or through membrane-associated channels (such as OSCA, DEK1, or PIEZO)
605 (Landrein and Ingram, 2019; Codjoe et al., 2021; Fobis-Loisy and Jaillais, 2021). To our
606 knowledge, fluorescent sensors derived from potential mechanosensors have not yet been
607 developed. Alternatively, it has also been shown that cortical microtubules robustly respond to

608 the application of mechanical forces in a variety of plant organs (Hamant et al., 2008;
609 Sampathkumar et al., 2014; Robinson and Kuhlemeier, 2018) and it has even been hypothesized
610 that microtubules themselves may act as mechanosensors (Hamant et al., 2019b). Mechanical
611 forces can thus be probed by measuring the level of organization and orientation of cortical
612 microtubules by confocal microscopy. This method has been applied to the seed, where the
613 repartition of forces within the layers of the outer-integument (outermost layers of the seed
614 coat) was assessed by comparing microtubule organization with the responses to forces within
615 these layers (Creff et al., 2015). However, it is important to note that microtubules can also
616 respond to other signals such as light and hormones; thus, their organization may not only be
617 linked to mechanical stress patterns (Landrein and Hamant, 2013).

618
619 Mechanical forces can also be assessed by quantifying the expression of fluorescent reporters
620 for mechanosensitive genes such as *ZINC FINGER PROTEIN2* in stems, *SHOOT MERISTEM*
621 *LESS* in meristems, and *EUI-LIKE P450 A1* in seeds (Martin et al., 2014; Creff et al., 2015;
622 Landrein et al., 2015). This approach was recently applied to developing seeds to assess stress
623 levels in a mutant impaired in turgor pressure (Creff et al., 2021). However, this type of analysis
624 is limited by the fact that the mechanosensitive genes that have been characterized to date are
625 only expressed in a small subset of cells in specific tissues. Finally, mechanical perturbations
626 have been shown to trigger rapid changes in intracellular calcium levels, apoplastic reactive
627 oxygen species production, and apoplastic pH (Monshausen et al., 2009). These responses can
628 be monitored using specific fluorescent reporters imaged by confocal microscopy (Martinière
629 et al., 2018; Li et al., 2019a; Nietzel et al., 2019). This method has notably been used in the
630 shoot apical meristem where it was shown that the response of the auxin transporter PIN1 to
631 mechanical forces relies on a transient Ca^{2+} response that could be monitored using the
632 fluorescent reporters R-GECO1 and GCaMP6f (Li et al., 2019a). These sensors are thus very
633 promising tools to study the rapid responses of cells to mechanical perturbations. However, it
634 remains to be shown if they can also be used to assess internal stress levels, notably during
635 growth, which happens on slower timescales.

636

637 **The rise of single cell approaches to study mechanics**

638 These last couple of years have seen the rapid development of single cell approaches, including
639 live-cell imaging methods. In developmental mechanobiology, single-cell systems represent a
640 simpler model to study the role of mechanical forces *in cellulo*, avoiding the additional
641 complexity brought about by the tissue context (e.g., chemical signals, impact of neighboring

642 cells, complex mechanical stress patterns). Recent studies have used such single cell approaches
643 to assess the relative contributions of both cell geometry and cortex tension to cortical
644 microtubule behavior (Colin et al., 2020; Durand-Smet et al., 2020). In these studies, wall-less
645 plant cells, also called protoplasts, were confined in microfabricated wells of various shapes
646 and sizes (**Figure 4A**). The protoplasts were either placed under hyperosmotic conditions (i.e.,
647 with a reduced cortex tension) or under hypoosmotic conditions (i.e., with an increased cortex
648 tension), and cortical microtubule orientation was then analyzed (**Figure 4B and C**). In cells
649 confined in rectangular microwells and exhibiting a reduced cortex tension, cortical
650 microtubules tended to align with the long axis of the cell (Colin et al., 2020; Durand-Smet et
651 al., 2020). By contrast, using microwells of a similar shape, in cells with an increased cortex
652 tension, cortical microtubules mainly aligned with the shortest axis of the cell, which also
653 corresponds to the principal stress direction in these cells (Colin et al., 2020).

654

655 One of the main features of protoplasts is their capacity to regenerate a whole organism from a
656 single cell. To investigate this process, microfluidic-based systems were recently designed and
657 adapted to follow protoplast development. In these systems, protoplasts are trapped in
658 chambers, where they are immobilized. Contrary to the previous system, nutrient medium can
659 circulate between chambers, allowing long-term kinetic experiments to be performed. Using
660 such a system combined with a microscope set-up, a recent study investigated the influence of
661 photoperiod on the growth of the moss *Physcomitrella patens* (Sakai et al., 2019). By adding
662 hormones to the circulating medium, the authors also observed the induction of leafy buds
663 (**Figure 4D**)(Sakai et al., 2019). In another study, a microfluidic platform was designed with
664 microelectrodes, coupled with electrical impedance spectroscopy, to study primary cell wall
665 regeneration at the single-cell level (Chen et al., 2020) (**Figure 4E**). In this study, cells
666 displaying a completely regenerated cell wall exhibited higher impedance values (i.e. dielectric
667 properties) compared to nascent protoplasts (Chen et al., 2020). This system also allows
668 researchers to discriminate between several cell wall mutants and wild-type cells, thus
669 providing a new tool for phenotypic analyses (Chen et al., 2020).

670

671 There are a number of limitations to such approaches. For example, the cell wall of a protoplast
672 regenerates but may have different properties compared to that of cells in a multicellular
673 context. In addition, it is not clear that properties deduced from experiments on individual
674 isolated cells can be easily applied to cells in their native tissues and organs. Thus, data obtained
675 from single cell experiments should be backed-up by in vivo analyses, when possible.

676 Furthermore, slight differences in the experimental design may influence the physico-chemical
677 environment of the cells and ultimately have strong impacts on the conclusion. However, this
678 also represent an opportunity. Indeed, if unexpected differences are found, researchers can take
679 advantages of minimal systems, since they are fully controlled, to understand which variables
680 differentially affected the results. Altogether, these single-cell approaches, combined with live-
681 cell imaging and microfluidic methods, open new opportunities to test biological hypothesis in
682 a highly-controlled manner.

683

684 **Key challenges for live plant cell imaging and possible solutions**

685

686 **Inherent difficulties in imaging plant cells**

687

688 *Apoplast*

689 The plant apoplast (i.e. space outside the plasma membrane) can be described as a
690 “microscopist’s nightmare”, as it represents one of the most formidable challenges for plant
691 cell biologists in terms of imaging. Indeed, plant cells are embedded in a thick cell wall often
692 made of highly autofluorescent and impermeable materials. The presence of thick cell walls
693 limit observations of the plasma membrane and the cell cortex in TIRF microscopy.
694 Nonetheless, TIRF/VA-TIRF microscopy has been successfully used to study plant tissues with
695 relatively thin walls, such as the root elongation zone, root hairs, hypocotyls, and young leaves
696 (Konopka et al., 2008; Konopka and Bednarek, 2008; Gronnier et al., 2017; Johnson and Vert,
697 2017; Platre et al., 2019; Johnson et al., 2020; Narasimhan et al., 2020; Smokvarska et al., 2020;
698 Bayle et al., 2021; Johnson et al., 2021). Plant cell biologists also have to face a sometimes
699 impermeable apoplast, which drastically limits the possibility to exogenously add fluorescent
700 compounds. This is one reason why the field is heavily dominated by the use of genetically
701 encoded constructs and fluorescent proteins. The apoplast is also a highly acidic environment,
702 which strongly affects most fluorescent proteins. The solution is to use pH resistant fluorescent
703 proteins, but established sensors that work in the cytoplasm, such as hormone or calcium
704 sensors, may have to be re-engineered to work in such an environment. The apoplast can be
705 very rich in proteases, which often destabilize proteins. A possible solution would be to remove
706 cryptic protease target sites in synthetic reporters. However, in many cases, such sites are not
707 precisely known, which largely preclude such strategies at the moment.

708

709 *Autofluorescence*

710 Plant cells and tissues are very rich in pigments and are highly autofluorescent. In addition, the
711 autofluorescence due to phenolic and carotenoid compounds, as well as chlorophyll and
712 chromophores, spans a wide range of wavelengths. This high autofluorescence can mask true
713 signals, often decreases the signal-to-noise ratio, and complicates automated image analyses.
714 One solution is to use tissues with minimal autofluorescence. For example, root tip cells do not
715 have chlorophyll and also have less autofluorescence in their cell walls because they are
716 undifferentiated. Alternatively, it is possible to use spectral unmixing or fluorescence lifetime
717 imaging to separate true signals from autofluorescence.

718

719 *Cytoplasmic streaming*

720 Plant cells have very active cytoplasmic streaming, which means that intracellular trafficking
721 is fast and difficult to follow using fluorescence microscopy. To circumvent this problem, it is
722 possible to image undifferentiated cells, which have weaker cytoplasmic streaming than highly
723 differentiated cells. Another solution, which was recently introduced to study endocytosis, is to
724 reduce the dynamics of the system by rapidly lowering its temperature (Wang et al., 2020;
725 Johnson et al., 2021). Finally, one can also use fast imaging systems such as spinning disk
726 confocal microscopy, TIRF microscopy, or light sheet microscopy (**Table 1**).

727

728 **A quest to image plant cells and tissues in their native conditions**

729

730 *Gravity*

731 Plants grow according to the gravity vector, with positive gravitropism for the root and negative
732 gravitropism for the shoot. This makes it difficult to image certain parts of the plant over a
733 longer period of time, for example when imaging the root tip. In most microscope set-ups, the
734 slides are mounted horizontally, which blocks the gravitropic response. The use of vertical stage
735 microscopes, which allow roots to grow along the gravity vectors, facilitates the dynamic
736 analyses of cell division, as well as studying the gravitropic response and cell elongation (von
737 Wangenheim et al., 2017; Fendrych et al., 2018; Marhava et al., 2019; Serre et al., 2021).
738 Alternatively, light sheet microscopy also allows roots to grow vertically while performing live
739 imaging (von Wangenheim et al., 2016; Ovečka et al., 2018).

740

741 *Light*

742 Plants need light to develop, and they use signaling pathways/photoreceptors to respond to
743 many wavelengths of light. The use of laser beams to excite fluorescent proteins often also

744 triggers these signaling pathways. This is particularly problematic when studying light
745 responses but may also confound other results. It is thus important to carefully confirm that a
746 given response is not affected by the imaging conditions. Interestingly, yellow fluorescent
747 proteins are excited by green light (around 514 nm), a wavelength that plants are mostly blind
748 to. YFP derivatives have thus proven to be highly popular among plant biologists. Another
749 solution is to use highly sensitive microscopy techniques to limit the amount of light treatment
750 and thus the activation of light-sensitive pathways (i.e. spinning disk confocal microscopy, light
751 sheet fluorescence microscopy – see **Table 1**). Finally, for very long imaging experiments, it
752 may be necessary to add lighting above the microscope stage to mimic the day/night cycle.

753

754 *Soil and air*

755 The root system naturally grows in heterogeneous soil, while the aerial parts of the plant grow
756 in the air. However, plant biologist usually mount their plants in homogenous medium, most
757 often liquid or agar-based. Transparent soil solutions exist (Ma et al., 2019a), and microfluidic
758 devices are becoming increasingly diverse to mimic particular growth conditions, even when
759 heterogeneous (Stanley et al., 2018; Guichard et al., 2020; Yanagisawa et al., 2021). However,
760 this is clearly an area that needs to be further developed in the future. In particular, imaging
761 samples in the air is difficult, as the samples can dry out and the microscope objectives are often
762 not adapted for this type of imaging.

763

764

765 **Conclusions and future prospects**

766

767 The examples described above illustrate that live imaging of plant cells is challenging on
768 multiple levels. There is no perfect set-up: live imaging experiments always involve a series of
769 compromises. For example, it is beneficial to have very bright labeling in order to limit
770 photobleaching and generate images with high contrast. For genetically encoded reporters,
771 bright labeling is often associated with strong expression levels. However, strong expression of
772 such probes can deeply perturb the system under study. Thus, one should strike a delicate
773 balance between expression and sensitivity. There is, of course, room for improvement. New
774 fluorescent reporters should be developed that are less toxic, fully reversible, with better
775 dynamic range, and more quantitative. Very often, the development of such tools is highly
776 empirical. They necessitate significant investments in terms of wet lab experiments and can

777 take years of work, with no guarantee of success. However, when new sensors or reporters
778 become available, they can tremendously benefit their fields of study. The use of molecular
779 simulation and in vitro protein evolution are starting to boost the rational design of sensors. We
780 thus envision that the building of new genetically encoded reporters, as well as the production
781 of new dyes, will significantly enhance our ability to image various aspects of plant cell biology
782 in the future.

783

784 The choice of a microscopy technique is also a matter of compromise to match the method with
785 the spatio-temporal scale of the system under study. However, it has become increasingly clear
786 that many biological phenomena happen at multiple scales that impose feedback on each other.
787 It is likely that plant biologists will increasingly need “scale-bringing” technologies able to
788 image biological systems at multiple scales. For example, such systems could combine super-
789 resolution capabilities with a wide field of view to study entire organs, or they may be able to
790 perform ultrafast imaging over long periods of time. With advances in electronics, particularly
791 the development of detectors and cameras that are extremely sensitive, such “scale-bridging”
792 technologies are becoming a reality (Clark et al., 2020). The imaging set-up should also allow
793 plants to grow in an environment that is as native as possible in term of light, growing medium,
794 temperature, orientation, the laser power received for imaging, and so on. Various microfluidic
795 devices tailored to the study of precise plant biology phenomena are already emerging
796 (Grossmann et al., 2018; Clark et al., 2020). Given the relatively low cost and high versatility
797 of microfluidic systems, we expect that they will become more and more common in plant live
798 imaging experiments.

799

800 Finally, it is clear that image analyses and quantifications are formidable problems that will
801 require multidisciplinary solutions. Some of these solutions may be widely applicable to many
802 projects, such as computational tools to analyze cytoskeleton properties or cell contours. By
803 contrast, in many cases, relevant image analyses will require dedicated scripts and algorithms
804 to answer specific biological questions. It will thus be imperative to build dedicated platforms
805 to host and index these scripts so they can be (re)used, improved, and modified.

806

807 In this era of quantitative biology, image analysis of live imaging experiments is often the
808 limiting factor. It will be imperative to train the next generation of plant cell biologists with this
809 in mind.

810

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815

816 **Author contributions**

817 L.C., S.B. and B.L. wrote the paragraph on imaging mechanical properties and responses to
818 forces, R.M-A. and T.V. wrote the paragraph on hormone sensors, M-C.C wrote the
819 paragraph on cytoskeleton imaging, Y.J. wrote the paragraph on imaging membranes, the
820 introduction and discussion. All authors edited the final manuscript. A.B. and B.L. prepared
821 Figure 1, Y.J. prepared Figure 2, all the Tables, R.M-A. prepared Figure 3 and L.C. prepared
822 Figure 4.

Microscopy technique	Principle	Advantages and limitations
Confocal laser scanning microscopy (CLSM)	The sample is scanned point-by-point by a focused laser beam (raster scanning), out-of-focus signal is removed by an adjustable iris (i.e. pinhole), and an image is built up pixel-by-pixel by collecting the emitted light via sensitive point detectors (e.g. PMTs).	Versatile technique, as it works with both thick and thin samples and with many different objective magnifications (i.e. variable pinholes), can produce thin optical sections, can spectrally separate different fluorophores, and the focused laser beam is compatible with photoactivation or targeted photobleaching. However, the application of an intense and focused laser beam can lead to photodamage and photobleaching, and scanning the entire sample in 3D is relatively slow.
Spinning disk confocal microscopy (SDCM)	Excitation light passes through a series of pinholes on a rotating disk so that only the imaged pixels are illuminated at a given time, out-of-focus light is also removed by those pinholes and light is collected in parallel on sensitive array detector(s) (e.g. EMCCD or sCMOS camera)	Faster and more gentle imaging than CLSM at the expense of z-resolution (i.e. optical section not as thin as with CLSM), difficult to perform spectral imaging, need additional dedicated equipment for photomanipulation. Not as versatile as CLSM because it has fixed pinholes that are not adjustable to various objective magnifications.
Total Internal Refraction Fluorescence Microscopy (TIRFM)	The laser beam hits the coverslip/medium interface at a critical angle, leading to its total refraction, which locally emits a shallow evanescent wave (~100–200 nm). As a result, only the portion of the cell in close contact with the coverslip is illuminated.	Because there is no out-of-focus light, TIRF microscopes can be coupled with highly sensitive cameras, thereby allowing very fast acquisition as well as single molecule imaging. TIRF microscopy increase the resolution in depth (basically determined by the thickness of the evanescent wave); however, this technique is limited to the cell cortex.
Variable Angle Epifluorescence Microscopy (VAEM/VA-TIRF)	Variation of the TIRF technique that uses a subcritical angle for the laser beam, which does not lead to total refraction, but instead partial (inclined) illumination of the sample.	VA-TIRF/VAEM is sometimes referred to as the “dirty” TIRF technique. It is a compromise between a deeper excitation into the sample and a less contrasted image.
Light sheet fluorescence microscopy (LSFM)	The whole field-of-view is illuminated by a laser light sheet (i.e. thin slice of light of a few hundreds of nanometers to few micrometers) perpendicularly to the direction of the detection.	LSFM is very rapid and gentle in terms of phototoxicity and photobleaching, thus it allows long term imaging, or fast 4D imaging. Like SDCM, LSFM cannot perform spectral imaging easily and needs additional dedicated equipment for photomanipulation. Sample mounting can be difficult and often highly specialized, which means that LSF microscopes are often dedicated to specific applications and not highly versatile.
Two-photon excitation microscopy (TPEM)	Simultaneous excitation of a fluorophore by two photons with longer wavelength than the emitted light. It typically uses tunable femtosecond pulsed laser with a raster scanning as in CLSM.	Two-photon microscopy is used for deep tissue imaging, as near infrared light minimize scattering in the tissue and only the fluorophores in the focal plan are activated. High laser energy can destroy the cell by over-heating, which is a potential drawback, but it can be used to generate very precise cell ablation deep in the tissue. Many dyes are excited by the same wavelength in TPEM, which can generate strong background and reduces the choice of fluorophores for multicolor imaging.
Photoactivated Localization Microscopy (PALM)	Super-resolution microscopy technique based on stochastic activation of photo-activatable fluorescent proteins, which allows their precise localization. Images are reconstituted by iterative cycles of activation, acquisition, and photobleaching.	PALM has a very high lateral resolution (~20–30 nm) and is a single molecule imaging technique (as such, it is often performed in TIRF, which is a very sensitive imaging technique). However, it is very slow because it requires iterative image acquisition, and the cells receives a lot of laser power (photodamage). It also requires dedicated transgenic lines expressing photo-activatable or photo-switchable fluorescent protein fusions, and multicolor imaging is limited. PALM also requires a lot of post-acquisition processing.
Structured illumination microscopy (SIM)	Super-resolution imaging technique that uses interference patterns created by a grid. It requires several images (with translations and rotations of the grid) and post-processing to compute a super-resolved image.	SIM roughly double the resolution limit of light microscopy (~120 nm laterally, 300 nm axially). It can be done in 3D and with multiple fluorophores and is compatible with classical fluorescent proteins. Because several images need to be acquired, it can be slow, it requires image post-processing and somewhat long illumination time (photobleaching). The increase in

		resolution is not as high as in PALM. Note that it can be coupled with TIRF (TIRF-SIM) to increase the contrast.
Super-resolution confocal live imaging microscopy (SCLIM)	Spinning disk microscopy with several paralleled array detectors and post processing (i.e. deconvolution).	SCLIM is equipped with three array detectors (i.e. cameras), and as such it is fast and can acquire several channels simultaneously, making it a solution of choice to study rapid processes such as membrane trafficking. However, it relies heavily on image post-processing, and the increase in lateral resolution is due to the deconvolution algorithm and is thus modest.
Stimulated Emission Depletion (STED) microscopy	Scanning of the sample by two different laser pulses: a first excitation pulse (excitation laser), and a second doughnut-shaped pulse (depletion laser) for the selective deactivation of the fluorophore. The focal spot is raster scanned, like in CLSM.	Lateral resolution of ~50–70 nm (>500 nm axially), can be rapid but in a small field-of-view, deep imaging compared with other super-resolution techniques (10–15 μ m deep) and does not require image post-processing. Has not been extensively used in live imaging in plants, likely due to high phototoxicity (high-intensity depletion laser) and photobleaching.
Fluorescence Recovery After Photobleaching (FRAP)	Technique used to study fluorescent molecule diffusion based on the bleaching of a population of fluorophores and the subsequent quantitative analysis of the fluorescence recovery.	FRAP is a popular technique to study molecule diffusion because it can be performed on most CLSM and with standard fluorescent protein fusions. It provides information on the diffusion of an ensemble of molecules, but diffusion coefficient calculation requires complicated models (and thus is quite indirect).
Single particle tracking (SPT)	Technique aiming at tracking single fluorescent particles (e.g. single molecules or single objects such as vesicles or microtubule tips) to analyze their dynamics. Can be coupled with PALM (i.e. sptPALM) to obtain super-resolved localization of diffusing individual molecules.	SPT techniques are a direct measure of diffusion and they tend to be very accurate for relatively slow diffusing molecules/structures compared to other techniques. They rely on complex image post-processing: automated tracking algorithms. These algorithms work well only if individual structures are well-defined/isolated from each other.
Fluorescence Resonance Energy Transfer (FRET)	Energy transfer between a donor and acceptor fluorescent protein that happens when they are in close proximity (i.e. less than 10 nm) and at the correct orientation with respect to each other.	FRET is typically used as a ruler to study molecular proximity, for example to study protein-protein interactions, or intramolecular conformational changes in the case of ratiometric biosensors. It is a very powerful technique, as it can detect dynamic molecular interactions in vivo. FRET can be measured on a variety of microscopes (including CLSM and widefield microscopy). However, it is difficult to accurately measure in practice. In addition, it is difficult to predict a priori how well FRET will work between two interacting molecules, and it has to be tested empirically.
Fluorescence Lifetime Imaging Microscopy (FLIM)	Technique based on the exponential decay rate of a fluorophore, which requires the use of a pulsed illumination source.	FLIM is often used to accurately measure FRET, since the fluorescent lifetime of the donor decreases upon energy transfer. It can also be used to differentiate fluorophores with otherwise overlapping spectra and can (for example) help to filter out autofluorescence. Although they are becoming more and more accessible, most FLIM systems are complex to use both in terms of image acquisition and analyses.

Table 1. Light microscopy techniques described in this review.

Cytoskeleton	Sensor name	Sensor type	Construct	Comments	Ref. of transgenic line	NASC stock #
Actin	AtFim1	Actin binding	Full-length AtFim1	Induce morphological defect at high expression	Wang et al., 2004; Sheahan et al., 2004	-
	fABD2	Actin binding	C-terminal half of AtFim1 (aa 325–687)	Induce morphological defect at high expression	Wang et al., 2004; Sheahan et al., 2004; Ketelaar et al., 2004;	N799991
	LifeAct	Actin binding	Actin-binding peptide (17 aa) of yeast abp140p	Induce morphological defect at high expression	Era et al., 2009	-
Microtubule	MBD	Microtubule binding	human Microtubule Associated Protein-4 MAP4	Enhanced microtubule polymerization, nucleation, bundling, and stabilization	Marc et al., 1998	N799990
	MAP65-1	Microtubule binding	Microtubules Associated Protein of 65 kDa-1	Enhanced microtubule polymerization, nucleation, bundling, and stabilization	Lucas et al., 2011	N67830
	TUA6	Direct microtubule labeling	TUBULIN alpha 6 gene	Microtubule and cytoplasmic localization	Ueda et al., 1999	N6551
	TUA5	Direct microtubule labeling	TUBULIN alpha 5 gene	Microtubule and cytoplasmic localization	Liu et al., 2016	-
	TUB6	Direct microtubule labeling	TUBULIN beta 6 gene	Microtubule and cytoplasmic localization	Nakamura et al., 2004	N6550; N67065; N67065
	EB1	Plus-end microtubule tip	Arabidopsis End-Binding Protein-1a	Plus end tip of the growing microtubules	Chan et al., 2003	-

Table 2. Commonly used cytoskeleton markers in *Arabidopsis thaliana*. Table listing some of the commonly used genetically encoded cytoskeleton markers. Fim, FIMBRIN-LIKE; ABD, actin binding domain; MBD, microtubule binding domain; MAP, MICROTUBULE ASSOCIATED PROTEIN; TUA, TUBULIN alpha; TUB; TUBULIN beta; EB1, END-BINDING1

PM marker	PM targeting	Number of amino acids	Topology/ orientation	Comments	Ref. of transgenic line	NASC stock #
Lti6b (RCI2b / 29-1)	2 TM	54	Both termini are oriented toward the cytosol	From Ehrhardt GFP-fusion line collection	Cutler et al., 2000	N84726
Lti6a (RCI2a / 37-26)	2 TM	54	Both termini are oriented toward the cytosol	From Ehrhardt GFP-fusion line collection	Cutler et al., 2000	N84758
PIP2;1 (PIP2a)	6 TM	287	Both termini are oriented toward the cytosol	From Ehrhardt GFP-fusion line collection	Cutler et al., 2000	N84725
PIP1;4 (W138)	6 TM	287	Both termini are oriented toward the cytosol	From wave line collection	Geldner et al., 2009	N781666; N781687; N781708
NPSN12 (W131)	1 TM	265	N-terminus in the cytosol	From wave line collection	Geldner et al., 2009	N781665; N781686; N781707
SYP122	1 TM	341	N-terminus in the cytosol		Assaad et al., 2004	-
FH6	1 TM	899	C-terminus in the cytosol		De Rybel et al., 2010	-
KA1	Anionic lipid binding	50	Extrinsic protein translated in the cytosol	KA1 domain of human MARK1 protein	Simon et al., 2016	N2107345
Myr	lipid anchor: myristoylation	8	Facing the cytosol	First 8 AA of LeCPK1 must be located at the N-terminus	Willis et al., 2016	-
MAP (MP)	lipid anchor: myristoylation and palmytoylation	12	Facing the cytosol	First 12 AA of AtGPA1 must be located at the N-terminus	Martinière et al., 2012	-
8K-Farn	Lipid anchor + anionic lipid binding: prenylation + cationic peptide	18	Facing the cytosol	Last 18 AA of human K-Ras4B, must be located at the C-terminus	Simon et al., 2016	N2017343
GPI	Lipid anchor: glycosylphosphatidylinositol	87	Facing the apoplast	AA318 to 405 of AtAGP4, must be located at the C-terminus	Martinière et al., 2012	-

Table 3. Fluorescent plasma membrane markers commonly used to label and segment cell contours in *Arabidopsis thaliana*. PM, plasma membrane, TM, transmembrane region. Lti6, LOW TEMPERATURE INDUCIBLE; RCI, RARE-COLD INDUCIBLE; PIP, PLASMA MEMBRANE INTRINSIC PROTEIN; NPSN, NOVEL PLANT SNARE; SYP, SYNTAXIN OF PLANT; FH, FORMIN HOMOLOGY; KA1, KINASE-ASSOCIATED domain

Lipid	Sensor name	Sensor type	Localization in root tip	Comments	Ref. of transgenic line	NASC stock #
PI3P	PX ^{P40} (P3)	Translocation	late endosome / tonoplast		Simon et al., 2014	N2105606; N2105615; N2105623
	2xFYVE ^{HRS} (P18)	Translocation	Late endosome / tonoplast		Vermeer et al., 2006, Simon et al., 2014	N2105611; N2105620; N2105626
PI4P	1xPH ^{FAPP1} (P5)	Translocation	PM (++) / TGN (+) / cell plate (+++)	Coincident detection of PI4P and ARF1	Vermeer et al., 2009, Simon et al., 2014	N2105607; N2105616; N2106624
	2xPH ^{FAPP1} (P21)	Translocation	PM (+++) / weak TGN / cell plate (+++)	High affinity sensor	Simon et al., 2014	N2105612; N2105621
	3xPH ^{FAPP1}	Translocation	PM (+++) / occasional TGN / cell plate (+++)	High affinity sensor	Simon et al., 2016	-
	1xPH ^{FAPP1-E50A}	Translocation	PM (+++) / occasional TGN / cell plate (+++)	ARF1-binding site mutated	Ito et al., 2021 Nat Comm	-
	P4M ^{SIDM}	Translocation	PM / cell plate (+++)		Simon et al., 2016	N2017346
PI(4,5)P₂	1xPH ^{PLC} (P14)	Translocation	weak PM / cytosol	Low affinity	Vincent et al., 2005, Vermeer et al., 2007, Simon et al., 2014	N2105609; N2105618; N2105625
	2xPH ^{PLC} (P24)	Translocation	PM / cytosol	High affinity	Simon et al., 2014	N2105613; N2105622
	TUBBY-C (P15)	Translocation	PM / cytosol + nucleus		Simon et al., 2014	N2105610; N2105619
PI(3,5)P₂	2xML1N	Translocation	Late endosome (≠PI3P endosome)		Hirano et al., 2017	-
PA	1xPASS	Translocation	Weak PM / cell plate		Platre et al., 2018	N2107781
	2xPASS	Translocation	PM / cell plate / nucleus	High affinity	Platre et al., 2018	N2107782
	PAleon	FRET, ratiometric	Constitutive targeting at PM	Ratiometric / quantitative	Li et al., 2019	-
PS	C2 ^{Lact}	Translocation	PM / cell plate / endosomes / tonoplast		Simon et al., 2016 ; Platre et al., 2018	N2117347; N2107778
	2xPHE ^{VCT2}	Translocation	PM / cell plate / endosomes / tonoplast		Platre et al., 2018	N2107779; N2107780
DAG	1xC1a ^{PKC}	Translocation	Mostly cytosolic / PM / cell plate / TGN		Vermeer et al., 2017	-

	2xC1a ^{PKC}	Translocation	Cytosol / PM / cell plate / TGN	High affinity	Vermeer et al., 2017	-
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Table 4. Commonly used anionic lipid sensors in *Arabidopsis thaliana*. FAPP1, Four-phosphate-adaptor protein 1; HRS, Hepatocyte growth factor-regulated tyrosine kinase substrate; PLC, Phospholipase C; ML1N, cytosolic phosphoinositide-interacting domain (ML1N) of the mammalian lysosomal transient receptor potential cation channel, Mucolipin 1 (TRPML1); PASS, PA biosensor with superior sensitivity; Lact, Lactadherin; EVCT2, EVECTIN2; PKC, Protein Kinase C.

Hormone	Sensor name	Sensor type	Comments	Ref. of transgenic line	NASC stock #
Auxin	DR5	Transcriptional	9 inverted repeats of <i>TGTCTC</i>	Ulmasov et al., 1997	N9402, N9361, N799364, N2106112, N2106143, N2106173
	DR5v2	Transcriptional	9 inverted repeats of <i>TGTCCG</i>	Liao et al., 2015	N2105636
	DII-VENUS	Degradation	Domain II of IAA28 fused to fast-maturing yellow fluorescent protein VENUS	Brunoux et al., 2012	N799173
	R2D2	Degradation, ratiometric	Ratiometric expression of DII-3xVENUS and mDII-ntdTOMATO from two RPS5A promoters	Liao et al., 2015	N2105637
	qD2	Degradation, ratiometric	Ratiometric expression of DII-VENUS and TagBFP from a single RPS5A promoter	Galvan-Ampudia et al., 2020	-
	AuxSen	FRET, ratiometric	Engineering of tryptophan sensor to recognize auxin	Herud-Sikimic et al., 2021	N2110798–N2110801
GA	RGAm ^{PFYR}	Degradation, ratiometric	GA responsive DELLA without its regulatory function in transcriptional response	Shi et al., 2021	-
	GPS1	FRET, ratiometric	Based on GID1/GAI interaction	Rizza et al., 2017	-
ABA	6xABRE-R	Transcriptional	6xABRE element from <i>RD29A</i>	Wu et al., 2018	N71620
	6xABRE-A	Transcriptional	6xABRE element from <i>ABI1</i>	Wu et al., 2018	N71619
	ABACUS	FRET, ratiometric	Based on PYL1/ABI interaction	Jones et al., 2014	-
	ABAlleon	FRET, ratiometric	Based on PYR1/ABI1 interaction	Waadt et al., 2014; Waadt et al., 2020	-
	SNACS	FRET, ratiometric	Sensors of OST1/SnRK2.6 activity, based on 14-3-3/AKS1 interaction	Zhang et al., 2020	-
CK	TCS	Transcriptional	6 direct repeats of type B ARR-binding (A/G)GAT(T/C) element	Müller and Sheen 2007	N69181, N23900, N66322
	TCSn	Transcriptional	Tandem head-to-head and tail-to-tail orientations of type B ARR-binding (A/G)GAT(T/C) element	Zürcher et al., 2013	N69180
	TCSv2	Transcriptional	Alternating head-to-head and tail-to-tail orientations of type B ARR-binding	Steiner et al., 2020	-

			(A/G)GAT(T/C) element		
JA	Jas9-VENUS	Degradation	Jas domain of JAZ9 fused to the fast maturing VENUS-N7	Larrieu et al., 2015	N2105629
SLs	Strigo-D2	Degradation	Truncated domain of AtSMXL6 (AA 615 to 979) fused to fast maturing mVENUS	Song et al., 2021	-

Table 5. Genetically encoded hormone sensors available as stable *Arabidopsis thaliana* transgenic lines. Note that we referenced only reporters that have been engineered to act as biosensors in the sense that they represent minimal systems to report on hormonal activities. We thus excluded from this table full-length hormone-responsive promoters or proteins (that can be degraded or change localization upon hormone signaling), since they are more likely to be regulated by additional cues and to modify the system they are supposed to monitor.

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825 **References**

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1678 **Figure Legends:**

1680 **Figure 1. Examples of image analysis using the developing Arabidopsis seed as a model.**
 1681 A) Analysis of microtubule organization (MAP65-1-RFP) in a developing Arabidopsis seed at
 1682 2 DAP (days after pollination) with FibriTool and MorphographX (scale bar: 10 μm). The
 1683 orientation and length of the red bar represent the mean orientation and degree of organization
 1684 of the microtubule array in a given cell, respectively. B) Segmentation of a confocal stack of a
 1685 developing Arabidopsis seed (5 DAP) expressing LTi6b-GFP analyzed with MorphographX
 1686 (scale bars: 50 μm).

1687 **Figure 2. Principles of genetically encoded lipid biosensors.** A) Schematic representation of
 1688 “translocation” lipid sensors. Their localization alternates between membrane-bound and
 1689 cytosolic. Their membrane-bound fraction increases with increasing concentration of lipids, but
 1690 this can be difficult to quantify. B) Schematic representation of ratiometric FRET-based lipid
 1691 sensors, such as PAleon. They are more quantitative than translocation sensors, but are
 1692 constitutively targeted to a predetermined membrane. They can thus be used once the
 1693 membrane of interest has been identified (for example using translocation sensors).

1694

1695 **Figure 3. Design principles of different types of plant hormone sensors.** **A.** DR5, an
1696 example of a plant hormone transcriptional sensor. The DR5 auxin synthetic promoter contains
1697 9 repeats (violet arrows) of ARF TF binding sites that control the expression of a fluorescent
1698 protein (FP) in response to the hormone (green circle). **B.** qDII, an example of a plant hormone
1699 degradation-based sensor. The qDII ratiometric sensor is composed of two FPs: FP1, fused to
1700 a DII degron domain; and FP2, whose expression is controlled by the same constitutive
1701 promoter. Auxin triggers ubiquitination of the DII domain and the further degradation of FP1.
1702 This can be quantified using the FP2 signal, which remains constant, as a reference. **C and D.**
1703 FRET-based plant hormone sensors. Two types of FRET sensors are available. The auxin FRET
1704 sensor AuxSen (C) uses the dimer of TprR (*Escherichia coli* tryptophan repressor, in grey)
1705 fused to two FPs, the donor and acceptor, which come in close contact due to a conformational
1706 change that follows auxin binding to TprR. For ABACUS, ABAleon (ABA), and GPS1 (GA)
1707 FRET sensors (D), donor and acceptor FPs are fused to two protein interacting partners (light
1708 blue and yellow) that bind to each other in the presence of the hormone.

1709

1710 **Figure 4. Single cell approaches to study cellular responses to mechanical forces.** **A)**
1711 Schematic representation of the device used to confine protoplasts to microwells (adapted from
1712 Colin et al., 2020). Briefly, a drop of a solution containing a suspension of protoplasts is
1713 deposited into the microwells of an Ibidi dish (1). Close-up of microwells containing protoplasts
1714 in 600 mOSMOL mannitol solution (2). Once in microwells, the protoplasts are ready to be
1715 imaged (4). In this figure, protoplasts are pressurized using a hypo-osmotic solution (280
1716 mOsmol mannitol (3), as in Colin et al., 2020). Many other types of experiments can be done
1717 (microwell coating, cell division experiments, and so on). **B)** Microtubule signals (P35S:GFP-
1718 MBD) in deformed protoplasts confined in agar wells (*adapted from Durand-Smet et al., 2020*).
1719 Scale bar, 10 μm . **C)** Analysis of microtubule orientation (*adapted from Colin et al., 2020*).
1720 Example of microtubule signal (p35S:GFP-MBD) in a protoplast confined in a 15x20 μm
1721 microwell. The dotted red line represents the region of interest (ROI) in which cortical
1722 microtubule orientation has been performed (left). The orientation of cortical microtubules in
1723 each ROI is color coded (middle). Polar histograms represent the cortical microtubule angle
1724 distribution for the protoplast (right). Each bar corresponds to an angle range of 9°. Schematic
1725 representation of cortical microtubule orientations are indicated on the plot. **D)** Time-lapse
1726 recording of the development of leafy buds of *Physcomitrella patens* (*adapted from Sakai et*
1727 *al., 2019*). Arrow head indicate leafy buds. Scale bar, 70 μm . **E)** Microscope image of a trapped

1728 Arabidopsis mesophyll cell (*adapted from Chen et al., 2020*). Flow direction was from left to
1729 right (red arrows). The three coplanar microelectrodes are represented by parallel black thick
1730 lines. The middle electrode acts as the exciting electrode.
1731

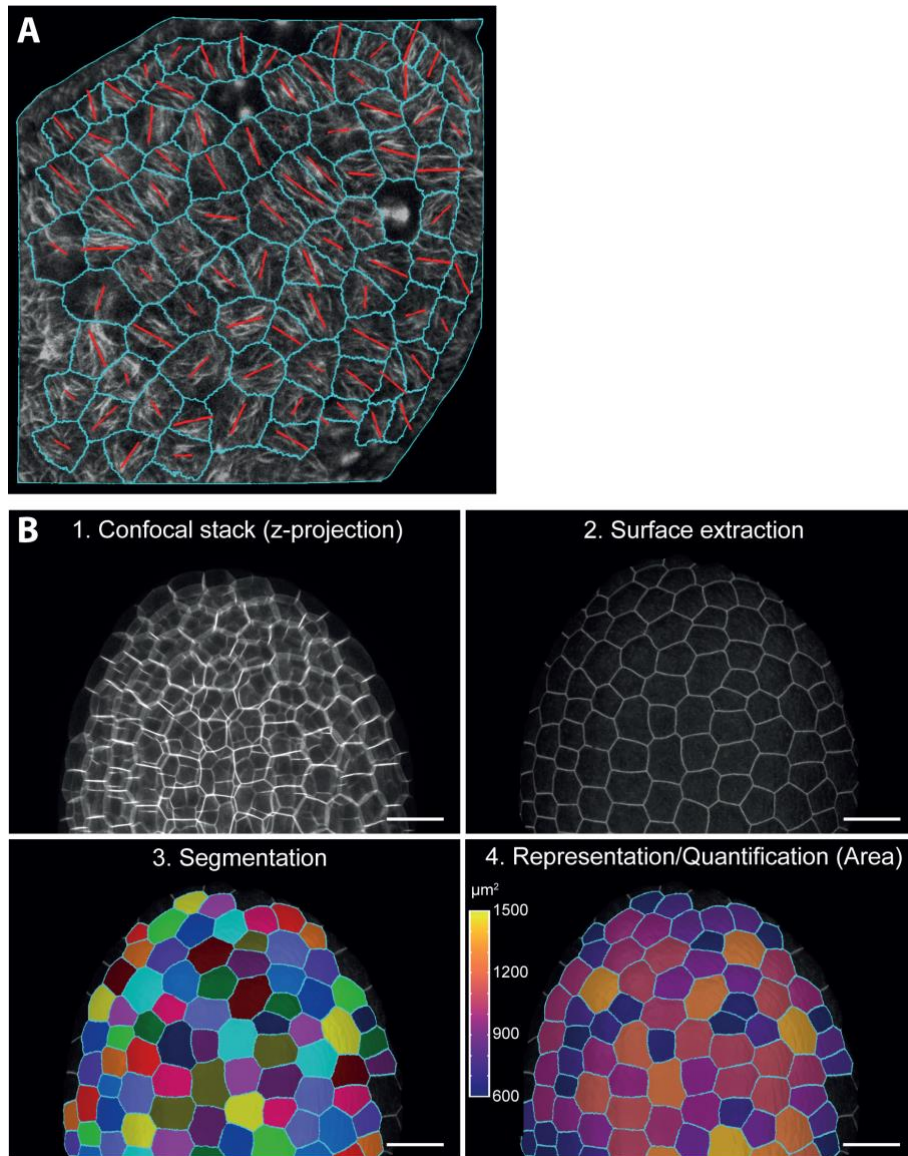


Figure 1. Examples of image analysis using the developing Arabidopsis seed as a model. A) Analysis of microtubule organization (MAP65-1-RFP) in a developing Arabidopsis seed at 2 DAP (days after pollination) with FibrTool and MorphographX (scale bar: 10 μm). The orientation and length of the red bar represent the mean orientation and degree of organization of the microtubule array in a given cell, respectively. B) Segmentation of a confocal stack of a developing Arabidopsis seed (5 DAP) expressing LTI6b-GFP analyzed with MorphographX (scale bars: 50 μm).

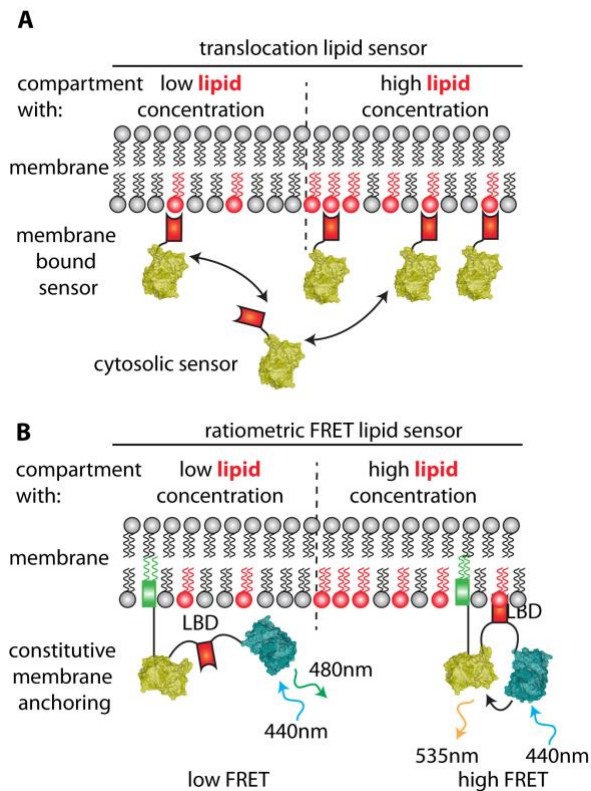


Figure 2. Principles of genetically encoded lipid biosensors. A) Schematic representation of “translocation” lipid sensors. Their localization alternates between membrane-bound and cytosolic. Their membrane-bound fraction increases with increasing concentration of lipids, but this can be difficult to quantify. B) Schematic representation of ratiometric FRET-based lipid sensors, such as PAleon. They are more quantitative than translocation sensors, but are constitutively targeted to a predetermined membrane. They can thus be used once the membrane of interest has been identified (for example using translocation sensors).

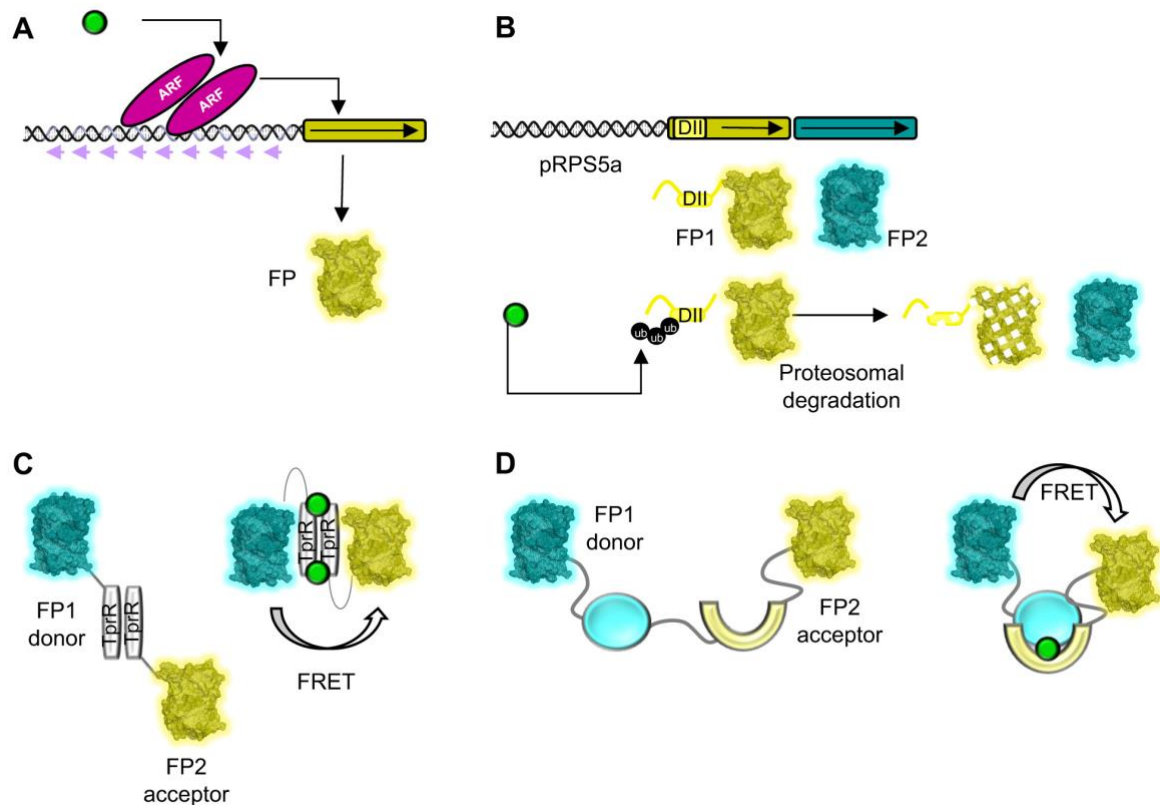


Figure 3. Design principles of different types of plant hormone sensors. **A.** DR5, an example of a plant hormone transcriptional sensor. The DR5 auxin synthetic promoter contains 9 repeats (violet arrows) of ARF TF binding sites that control the expression of a fluorescent protein (FP) in response to the hormone (green circle). **B.** qDII, an example of a plant hormone degradation-based sensor. The qDII ratiometric sensor is composed of two FPs: FP1, fused to a DII degron domain; and FP2, whose expression is controlled by the same constitutive promoter. Auxin triggers ubiquitination of the DII domain and the further degradation of FP1. This can be quantified using the FP2 signal, which remains constant, as a reference. **C** and **D.** FRET-based plant hormone sensors. Two types of FRET sensors are available. The auxin FRET sensor AuxSen (C) uses the dimer of TprR (*Escherichia coli* tryptophan repressor, in grey) fused to two FPs, the donor and acceptor, which come in close contact due to a conformational change that follows auxin binding to TprR. For ABACUS, ABAleon (ABA), and GPS1 (GA) FRET sensors (D), donor and acceptor FPs are fused to two protein interacting partners (light blue and yellow) that bind to each other in the presence of the hormone.

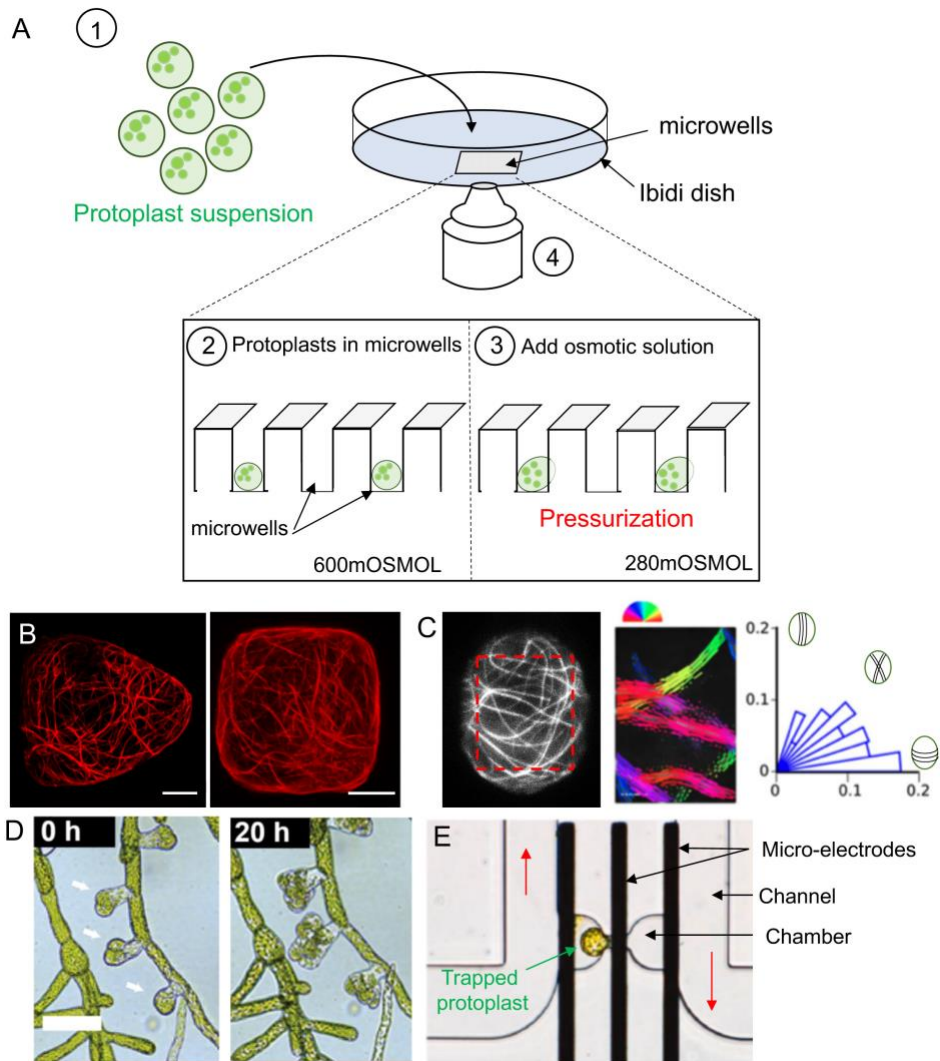


Figure 4. Single cell approaches to study cellular responses to mechanical forces. A) Schematic representation of the device used to confine protoplasts to microwells (adapted from Colin et al., 2020). Briefly, a drop of a solution containing a suspension of protoplasts is deposited into the microwells of an Ibidi dish (1). Close-up of microwells containing protoplasts in 600 mOSMOL mannitol solution (2). Once in microwells, the protoplasts are ready to be imaged (4). In this figure, protoplasts are pressurized using a hypo-osmotic solution (280 mOsmol mannitol (3), as in Colin et al., 2020). Many other types of experiments can be done (microwell coating, cell division experiments, and so on). B) Microtubule signals (P35S:GFP-MBD) in deformed protoplasts confined in agar wells (adapted from Durand-Smet et al., 2020). Scale bar, 10 μm . C) Analysis of microtubule orientation (adapted from Colin et al., 2020). Example of microtubule signal (p35S:GFP-MBD) in a protoplast confined in a 15x20 μm microwell. The dotted red line represents the region of interest (ROI) in which cortical microtubule orientation has been performed (left). The orientation of cortical microtubules in each ROI is color coded (middle). Polar histograms represent the cortical microtubule angle distribution for the protoplast (right). Each bar corresponds to an angle range of 9°. Schematic representation of cortical microtubule orientations are indicated on the plot. D) Time-lapse recording of the development of leafy buds of *Physcomitrella patens* (adapted from Sakai et al., 2019). Arrow head indicate leafy buds. Scale bar, 70 μm . E) Microscope image of a trapped Arabidopsis mesophyll cell (adapted from Chen et al., 2020). Flow direction was from left to right (red arrows). The three coplanar microelectrodes are represented by parallel black thick lines. The middle electrode acts as the exciting electrode.