From the identification of 'Cells', to Schleiden & Schwann's Cell Theory, to Confocal Microscopy and GFP lighting up the Plant Cytoskeleton, to Super-Resolution Microscopy with Single Molecule Tracking: Here's...

A Short History of Plant Science Chapter 5:

A Short History of Plant Light Microscopy

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When the microscope was first introduced to scientists in the 17th century it started a revolution. Suddenly a whole new world, invisible to the naked eye, opened up to curious explorers. In response to this realization Nehemiah Grew, one of the early microscopists, noted in 1682 '*that Nothing hereof remains further to be known, is a Thought not well Calculated.*'[1]. And indeed, with ever increasing resolution, there really does not seem to be an end to what can be explored with a microscope.

The Beginnings: Plant Internal Structures and 'Cells' (1600-1835)

While simple lenses were being used as magnifying glasses for several centuries, the early **17th century** brought the invention of the compound microscope, and with it launched the scientific field of microscopy[2]. It is not clear who invented the first microscope, but it was most likely developed from early telescopes[2]. Galileo Galilei built his first telescope in the early 1600s and used it to chart the stars[2]. He subsequently published his treatise 'Sidereus nuncius' **(1610)** about his observations[2,3]. However, Galileo also observed that he can use his telescope to magnify objects if he moved the lenses further apart[2]. It is conceivable, that this observation, probably made by others as well, has led to the first microscopes being developed[2]. One of the first documented microscope makers was Cornelius Drebbel, and Galileo built his first microscope based on a Drebbel microscope in the **mid 1620s**[2]. This microscope was used by Federico Cesi and Francesco Stellut to observe a bee and a beetle, which is possibly the earliest documented use of a microscope[2]. Simple compound microscopes of the mid 17th century were basically hollow metal tubes containing a convex lens at each end – the objective lens close to the object to collect and focus the light, and the eyepiece lens on the other end for additional magnification[2]. These earliest compound

microscopes allowed for magnifications of up to 25 times, but these were quickly improved in the following years. Robert Hooke and Antonie van Leeuwenhoek were two pioneering microscopists in those years. Antonie van Leeuwenhoek, a drapery salesman, was simply interested in finding a tool to better examine the quality of threads in the fabrics in his shop, which got him interested in lens making[4]. Eventually, he was able to create tiny lenses, which were able to magnify objects up to 250 times[4]. Robert Hooke, a polymath, had already been interested in optics and light refraction when he came across the new compound microscopes[5]. He too started to experiment with custom-made compound microscopes and self-made lenses to improve the quality of his instruments[5]. Eventually, both started documenting their microscopic work. Robert Hooke used his microscope to document everything, from microbes to plants, to man-made objects[6]. This resulted in the publication of Robert Hooke's book '*Micrographia: or Some Physiological Descriptions of Minute Bodies Made by Magnifying Glasses. With Observations and Inquiries Thereupon*' by the Royal Society of London in **1665**[6]. '*Micrographia*' became a bestseller, with Samuel Pepys, British politician and famous diarist, confiding to his diary that '*Before I went to bed, I sat up till 2 o-clock in my chamber, reading of Mr. Hookes Microscopical Observations, the most ingenious book that I ever read in my life.*'[4]. Antonie van Leeuwenhoek also read this book, and started to publish his own observations in the form of letters to the Royal Society in the late **1670s**[4,7]. Antonie van Leeuwenhoek focused mainly on insects and microorganisms, but did adventure a bit further as well. And so, in **1677**, he checked with the Royal Society of London if his latest work was publishable, writing *'If your Lordship should consider that these observations may disgust or scandalise the learned, I earnestly beg your Lordship to regard them as private and to publish or destroy them as your Lordship sees fit'*[8,9]. But the Society did consider van Leeuwenhoek's latest observations to be of scientific value, and so the first observation of sperm in human and animal ejaculate was published in **1678**[8,9]. From a plant microscopist's perspective, however, it is one figure that stands out among these earliest publications: In Robert Hooke's *Micrographia*, *Schem: XI, Fig: 1, A & B* shows a piece of cork (*click for Figure*)[6]. When examining this slice under his microscope, Hooke found that it had '*very little solid substance*', but was made up of little '*pores, or cells*'[6]. For Hooke, this observation demonstrated to him '*the true and intelligible reason of all the Phænomena of Cork*' - why it is so light relative to its size, why it floats on water, and why it is so springy when compressed[6]. But more important in retrospect is that this little sentence coined the word '*cell*' to describe cells[6].

The work of Robert Hooke and Antonie van Leeuwenhoek made them the "Fathers of Microscopy", and this new exciting field of research quickly got populated with other figures. One of the next big publications for the plant sciences came in **1682** with Nehemiah Grew's '*The anatomy of plants - with an idea of a philosophical history of plants, and several other lectures, read before the Royal Society*'[1]. This came at a time when it was not even accepted that plants were made up of organs, or had any internal structures at all. The book opens with a dedication to King Charles II that beautifully describes how the invention of the microscope forever altered our perception of the world, or rather, how it opened up a completely new world, which previously remained hidden to the human eye:

'*Your majesty will here see, that there are those things within a Plant, little less admirable, than within an Animal. That a Plant, as well as an Animal, is composed off several organical parts; some thereof may be called its Bowels. That every Plant has Bowels of diverse kinds, containing diverse kinds of liquors. That even a Plant lives partly upon air; for the reception whereof it has those Parts which are answerable to Lungs. So that a Plant is, as it were, an Animal in Quires; as an Animal is a Plant, or rather several Plants bound up into one Volume.*

Again, that all the said Organs, Bowels, or other Parts, are as artificially made; and for their Place and Number, as punctually set together; as all the Mathematic Lines of a Flower or Face. That the Staple of the Stuff is so exquisitely fine, that no Silkworm is able to draw anything near so small a thread. So that one who walks about with the meanest Stick, holds a Piece of Natures Handicraft, which far surpasses the most elaborate Needle-Work in the World.

In sum your majesty will find, that we are come ashore into a new World, whereof we see no end.'[1].

In the following book Grew systematically describes a plant's morphology and anatomy, covering seeds, leaves, stems, roots and flowers, always accompanied by beautiful illustrations of the entire organ, magnifications and cross-sections (*see plate XXXVI. B here*)[1]. At a time when it was not yet accepted that plants had any inner structures, let alone organs at all, his images showed that plants were indeed as complex as animals.

What the illustrations in these books also demonstrate, is that the authors not only had to be masters of microscopy, but also had to be great at sketching and drawing, to adequately document their observations. The days of cameras and detectors were still centuries away at

97 that point. In the early 1800s, however, an invention by William Wollaston did bring some
98 help. Thankfully for many microscopists coming after him, William Wollaston was, in his
99 own account, not good at drawing: '*Having* (...) *amused myself with attempts to sketch*
100 *various interesting views without an adequate knowledge of the art of drawing, my mind was*
101 *naturally employed in facilitating the means of transferring to paper the apparent relative*
102 *positions of the objects before me.*'. This led him to develop a device called the camera lucida
103 in **1807**[10]. The camera lucida is as simple as it is ingenious. A four-sided glass prism is placed
104 in front of the eye piece of the microscope and above the piece of paper where the drawing is
105 supposed to be made[10,11]. In the prism, two sides are arranged at a 135° angle to produce two
106 reflections of the light coming from the microscope through total internal reflection, thereby
107 producing a non-inverted or reversed image of the object under the microscope at the position
108 of the eye[10,11]. Since the prism is above the piece of paper, the microscopist sees both, the
109 reflected image at the edge of the prism, and the drawing surface in front of him, and can
110 sketch out the key points of the object onto the paper[10,11]. As the superimposed image and the
111 paper will not be in the same focal plane, a lens is additionally placed between the prism and
112 the paper, to bring both into the same focus[10,11]. The camera lucida, or similar devices, such as
113 Sömmering's mirror, were used well into the 20th century, and were instrumental in making
114 the microscope the powerful tool it has become for scientists[11].

115 While Nehemiah Grew's observations made it clear that plants were indeed made up of
116 several different structures, it was not yet clear how all these different structures are formed
117 and connected, and how Hooke's cells fit in. Between **1800** and **1810**, the French botanist
118 Charles-François Brisseau de Mirbel made his own microscopic observations of the anatomy
119 of different plants[12]. These eventually led him to the understanding that green plants are made
120 up of one tissue from a single continuous membrane, which envelopes and interconnects the
121 individual cells[12,13]. The individual cells, he argues, where made up from parenchyma, and
122 grow from, between or inside of older cells[12–14]. This hypothesis got Brisseau de Mirbel a lot
123 criticism from his contemporaries, who believed that cells were individual units, put together
124 to form a tissue, and eventually this disagreement led him to further investigations to prove
125 his point[14,15]. Going into this new work, he declared that '*Thirty years have passed since I*
126 *first published my opinions on several points. They were strongly attacked. Today now I want*
127 *to submit them to my own review: I will try to be impartial.*' **(1835)**[14]. He decided to focus on
128 a thorough investigation of one specific plant, rather than looking at several different ones for
129 his re-examination, and chose the liverwort *Marchantia polymorpha*, instead of a plant with a
130 stem, woody tissue, flowers and such, since '*it is the cellular tissue which I have chosen to*

131 *investigate, and, consequently, a whole plant made of this tissue is more suitable than any*
132 *other*'[14]. While Brisseau de Mirbel's view that the cellular tissue of plants is made from one
133 continuous membrane turned out to be wrong, and he acknowledged so, his work was still
134 important in understanding where cells came from, as he was among the first to hypothesize
135 that new cells arise somehow from older cells[15,16]. And furthermore, his description and
136 illustrations of *M. polymorpha* contributed to the introduction of this liverwort as a model
137 plant (*see Plate I here*)[15].

138 **Plant Cell Nuclei and Chromosomes, the Cell Theory and Cell Division (1830-1930)**

139 The next big step in understanding plant life via microscopy came in the mid-19th century at
140 the hands (and eyes) of Matthias Jacob Schleiden and Theodor Schwann[16]. Matthias
141 Schleiden already believed that an organism was made up of a society of cells, and so he
142 focused his work on discovering where these cells originated from. Brisseau de Mirbel's idea
143 that they somehow arise from older cells was not accepted yet, and cells were sometimes
144 suggested to just 'crystallize'[17]. Schleiden built his work in part on the finding of Robert
145 Brown, that all plant cells seem to have one nucleus **(1831)**[18]. So Schleiden came up with the
146 idea that this body was the potential starting block of a new cell. His first big discovery was
147 that the nucleus contained another, smaller granule, the nucleolus[19]. Then, while monitoring
148 the endosperm of palm seeds over time, he observed free-nuclear divisions of the endosperm
149 (*see Fig. 1 a-e here*)[19]. Such divisions occur before the first zygotic division in the endosperm
150 of the embryosac, resulting in 4 to 8 free nuclei before first cell walls are formed and the
151 nuclei are separated[20]. From these observations of an (as we now know) atypical cell-division
152 event that only occurs in the endosperm, he logically, but incorrectly, concluded that all new
153 cells are formed *de novo* around a nucleolus[19]. According to his hypothesis, the nucleus is first
154 formed around the nucleolus, which then starts to grow[19]. Once it has reached its full size, the
155 cell emerges from the nucleus as a bubble, and expands until it reaches its final size[19]. Then,
156 the cell wall is laid down and the cell is fully established **(1838)**[19]. In 1837, while preparing
157 his observations for publication, Schleiden met Theodor Schwann, his colleague at Johannes
158 Peter Müller's Institute at the University of Berlin, for dinner[16]. On this occasion, '*Schleiden,*
159 *this illustrious botanist pointed out to me the important role that the nucleus plays in the*
160 *development of plant cells*', Schwann recalled later[16]. He had just recently observed cells and
161 with nuclei in the notochord of toads, and following his dinner with Schleiden he also
162 observed the same in mammalian cartilage tissue[16,21]. Realizing these common principles
163 between plants and animals, Schwann proposed a general cell theory in **1839**[16,21]. Based on

164 his and Schleidens observations, Schwann defined a cell as consisting of a nucleus (with
165 nucleolus), and fluidic content constrained within a wall[21]. He further hypothesized that all
166 organisms, be it a plant, animal or human, are made up of one or more cells, with the cell
167 being the basic unit of structure and organization of an organisms[21]. Finally, he concurred
168 with Schleiden that new cells are formed *de novo* around the nucleus, which therefore
169 represented a common principle of development for all organic tissues[21]. This 'cell theory',
170 while not completely correct, led Edmund Wilson to remark in **1896** that "*no other biological*
171 *generalization, save only the theory of organic evolution, has brought so many apparently*
172 *diverse phenomena under a common point of view or has accomplished more for the*
173 *unification of knowledge*"[22]. It is therefore somewhat ironic that because the cell theory
174 remained so compelling as a generalized model for how all organic tissues form and develop,
175 it actually inhibited research into cell division for decades, due to its inclusion of the *de novo*
176 cell formation aspect[17]. Still, the finally accepted fact that new cells are formed via division of
177 existing cells was yet again based on the work of two plant microscopists: Hugo von Mohl
178 and Carl Nägeli[23]. Von Mohl, who was an expert for microscopy, as well as plant sample
179 preparation, and among the many phenomena he observed and documented were, e.g., the
180 formation, as well as the opening and closure of stomata[23–26]. In regards to cell divisions, von
181 Mohl actually observed and documented them in the algae *Cladophora glomerata* already in
182 1835 (see Fig. 3-5 *here*)[23]. With this work he also coined the term '*protoplasm*' to describe
183 the content of a cell. Von Mohl's observation was later supported by Carl Nägeli, who
184 observed cell division in pollen in 1842[25,27]. While the working hypotheses of von Mohl and
185 Nägeli were not accepted over the cell theory at the time, it did form the basis for subsequent
186 studies confirming that new cells are indeed formed by cell division.

187 With the nucleus and nucleolus at the central focus of Schleiden's and Schwann's work, the
188 20th century opened with some publications on the content of the nucleus: the plant
189 chromosomes. In **1907** *Arabidopsis* pioneer Friedrich Laibach completed his PhD by
190 determining the number of chromosomes in different plant species, among them *Arabidopsis*
191 *thaliana*[28]. *A. thaliana* was only featured in his complete thesis however, and was omitted
192 from the publication, as it was not regarded as important enough at the time[29]. Laibach
193 himself helped change this view in the years to come (See also 'A Short History of
194 *Arabidopsis thaliana* (L.) Heynh. Columbia-0'[29]). Following this work, Emil Heitz analysed
195 the chromosomes of liverworts in closer detail, thereby following in the footsteps of
196 Marchantia pioneer Brisseau de Mirbel and *Arabidopsis* pioneer Laibach **(1928)**[30,31]. Finding
197 density differences within the chromosomes during the telophase of mitosis, Heitz defined the

198 terms euchromatin and heterochromatin[30,31]. Both, Laibach and Heitz used the '*Abbe'scher*
199 *Zeichenapparat*', to document their work. This was an improved version of the camera lucida,
200 designed by Ernst Abbe for Zeiss microscopes[28,30].

201 Ernst Abbe may have pushed the boundaries for microscopists like hardly any other
202 individual person[32]. In the **1860s** Ernst Abbe joined Carl Zeiss in his newly founded Zeiss
203 Company as director of the research department, and later went on to become a co-owner of
204 the company in the **1870s**[32]. During his time at Zeiss he studied the theory of optics and
205 microscopy, and, based on his findings, started to develop and build better microscopes[32–34].
206 Some of his most important contributions to the field are the invention and implementation of
207 apochromatic lenses into microscopes to focus light of different wavelengths to the same
208 plane, the development of the first refractometer to determine the refractive indices of
209 different samples and media, a definition of the numerical aperture for an objective lens, and a
210 formula to define the resolution limit of a microscope[33–36]. When the first ZEISS logo was
211 issued in 1904 it featured the company's name inside a frame outlining Abbe's apochromatic
212 doublet lens, highlighting the importance of this invention[37]. Another important Zeiss
213 employee at that time was August Köhler. Köhler tackled another major problem of
214 microscopy at the time, which was the uneven illumination of the field of view, which in
215 addition often showed the illumination source (e.g. the light bulb filament) in the final
216 image[38]. Köhler developed the Köhler-illumination technique, which utilizes a collector lens
217 in front of the light source to defocus the light source (e.g. the bulb filament) from the sample
218 plane, thereby removing it from the image **(1893)**[38]. Additionally, an adjustable field
219 diaphragm is installed in front of the collector lens to get rid of any stray light[38]. Finally, a
220 condenser lens focuses the light onto the sample, thereby ensuring a homogenous illumination
221 of the entire field of view[38].

222 Thanks to the work of Abbe and Köhler, the general imaging conditions improved
223 dramatically for microscopists at the end of the 19th century. And there was another
224 development around the turn of the century that would radically change the way microscopists
225 work; photomicrography. Photomicrography had been invented and patented already in **1850**,
226 when Richard Hill Norris used it to image blood cells. But two important developments really
227 opened up the field of microscopy to photomicrography. The first was the aforementioned
228 Köhler-illumination in 1893, since a homogenously illuminated field of view is a prerequisite
229 to obtain a good photomicrograph. The second was the development of the Leitz Camera, or
230 LeiCa in short, in the early 20th century[39]. The Leica 1 was released as a portable and easy to

231 use camera in **1925**, and in combination with a microscope with Köhler-illumination finally
232 enabled scientists to take photos of their observations, rather than having to draw them[39].

233 **Plant Cell Organelles & the Cytoskeleton (1930-1980)**

234 The work of Ernst Abbe and August Köhler advanced the common light microscope to a point
235 where it's potential was almost exhausted. At this point, new microscopy techniques were
236 needed to increase the resolution and image quality further. The first such major improvement
237 came in **1934** when Frits Zernike published the theoretical work that eventually resulted in
238 phase-contrast microscopy (PCM) **1938**[40,41]. When light passes through a sample it is
239 scattered, resulting in changed phases of the light waves compared to the non-scattered
240 illumination light that did not pass through the sample[40,41]. These phase changes can be
241 converted into differences in brightness to enhance the contrast in the final image[40,41]. In a
242 phase contrast microscope this is achieved by filtering the non-scattered illumination light to
243 decrease its amplitude, and by changing the phase of the non-scattered illumination light to
244 match its phase with the phase of the scattered light, thereby creating constructive
245 interference[40,41]. This technique was especially important for biologists at the time, as it
246 increased the contrast, and hence the image quality of non-labelled samples[40,41]. And since
247 most samples were still unlabelled at the time, adding good contrast to the image meant a
248 giant leap forward. Accordingly Frits Zernike was awarded the Nobel Prize for Physics in
249 **1953** for his invention[42,43]. One early publication utilizing PCM in the plant field came in
250 **1955**, when Robert de Ropp analysed plant cells that he had cultured, trying to establish a
251 proper plant cell culture[44]. While he failed to establish a true cell culture as the cell protoplasts
252 steadfastly refused to divide in the culture medium employed, the improved contrast in his
253 images allowed him to not only see organelles in much closer detail, such as mitochondria
254 and moving nuclei, he could even observe the streaming of the cytoplasm, and he also
255 documented different stages of secondary cell wall formation (*see Fig. 7 here*)[44]. In the same
256 year Helen Sorokin documented mitochondria, stomata, and plastids clustered around the
257 nucleus in peeled lettuce epidermis cells (*see Fig. 2 here*), and also showed how Neutral Red
258 and Janus Green B can be used to stain mitochondria. For the latter, she also demonstrated
259 how the combination of PCM with vital stains can push the resolution even further[45].

260 Both, de Ropp and Sorokin, used PCM and photomicrographs to document their work, and
261 can therefore be considered state-of-the-art microscopists. However, there are always talented
262 people that push things a little further. Already ten years earlier, in **1946**, Henrik Lundegårdh
263 published his work on root hair development in wheat[46]. For this, he designed and built a

264 specialized experimental setup: First, he designed a little microfluidic chamber in which the
265 wheat seedling would grow in distilled water[46]. Through in- and outlets at each end of the
266 chamber he was able to run different solutions through it, and along the root of the growing
267 wheat plant[46]. This chamber was closed by a cover slip on top, and mounted onto a
268 microscope[46]. To document the reaction of the root hairs to different solutions washed
269 through the chamber, Lundegårdh had installed a film camera above the microscope with a
270 clock work to automatically run 32 mm film through the camera, and an automatic
271 electromagnetic shutter for the one second exposure time[46]. Using this setup, which preceded
272 the modern microfluidic platform RootChip[47] by 65 years, he was able to, among other things,
273 document that glucose accelerates hair growth, that a pH lower than 6 reduces growth, and
274 that the addition of auxin or calcium can counteract this negative effect, at least at a pH of 5[46].
275 Though educational videos of growing roots or emerging lateral roots had been recorded since
276 before the 1930s, this setup provided a whole new level of detail[46,48].

277 While plant microscopists were beginning to publish their work using PCM, Georges
278 Nomarski already further developed this technique into differential interference contrast
279 (DIC) microscopy **(1952-1955)**[49,50]. For DIC microscopy, two orthogonal polarized light rays
280 are used which both penetrate the sample slightly offset from each other, thereby experiencing
281 slightly different phase retardations, depending on the refractive index and thickness of the
282 sample at the point they pass through it[49,50]. Both rays are then re-combined but cannot fully
283 reproduce the initial polarization of the illumination light due to the subtle differences in
284 phase retardation experienced by both rays. A polarization filter oriented perpendicular to the
285 polarization of the illumination light is then used to reject the illumination light and transmit
286 specifically such light rays that penetrated through optically inhomogeneous parts of the
287 sample leading to a substantial increase in edge contrast[49,50]. This effort led to the
288 development of the ZEISS Nomarski System in **1965**. In **1966,** a prototype of this new DIC
289 microscope found its way into Robert Allen's Department of Biology at Princeton University,
290 and together with Andrew Bajer he created comparative images of *Haemanthus katheriniae*
291 (cape tulip) cells undergoing mitosis using either PCM or DIC[51]. Having demonstrated the
292 benefits of DIC microscopy for plant cells with this first paper, the pair immediately added a
293 second publication containing a time-series of DIC images following a cell undergoing
294 mitosis and cell plate formation (*see Fig. 2-7 here*)[52].

295 Helen Sorokin's use of Neutral Red and Janus Green B to stain mitochondria were the first
296 examples shown here for another new trend in the middle of the twentieth century. While

general stains have long been used, researchers now began to specifically develop and synthesize new stains. One of the new vital stains identified at that time was 4′,6-diamidino-2-phenylindole (DAPI), originally developed as a drug against Trypanosomiasis in **1971**[53]. It unfortunately failed as a drug, but in **1975** it was shown that it could be used to label DNA in the nucleus of cultured human cells, and a year later, in **1976**, it was shown to also work in plant cells[54,55]. Another important DNA stain set was the series of Hoechst stainings **(1975/1976)**[56,57]. Later on, more dyes for specific structures and organelles were added, such as 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)) to mark the plant endoplasmic reticulum **(1986)**[58]. And during the 1980s, the field of plant biology underwent a major revolution due to several developments, namely: the adoption of *Arabidopsis thaliana* as a model organism for the plant field, the establishment of plant transformation, and the identification of the cauliflower mosaic virus 35S promoter (see the Short History Chapters 1-3 for more on this plant science revolution[29,59,60]). In addition, these developments also brought the first genetically encoded reporter for plant light microscopy[61]. This came in the form of the *Escherichia coli β-glucuronidase* gene (*GUS*) **(1987)**[61]. The enzyme encoded by the *GUS* gene converts a colourless substrate (mostly X-Gluc) into the blue diX-indigo. Therefore, expression of *GUS* from a gene's specific promoter, will visualize the expression pattern of the investigated gene *in planta*[61].

Moreover, another important 'staining' method, immunofluorescence microscopy, was developed at the time to further improve the quality and resolution of images **(1974)**[62]. In the early 1930s researchers were able to purify and label pneumococcus antibodies, despite not even knowing for sure if these antibodies were proteins, or substances of a completely different nature[63]. This lead Albert Coons to test if he could use fluorescently labelled pneumococcus antibodies to actually locate antigens in tissue infected by pneumococcus[64]. By 1941, in the midst of World War II, Coons and his colleagues had managed to synthesize a fluorescein-antipneumococcal antibody, and were indeed able to stain pneumococcal antigens in the liver of an infected mouse[65]. Unfortunately, as mentioned by Coons concerning this breakthrough, '*I joined the Army in April, 1942, and the paper was written on a cross-country train. It was carefully re-written by Enders, who sent it off to the Journal of Immunology where it appeared in November, 1942. In the press of events, however, he forgot to send me a reprint, and I had no idea of its fate for many months. Finally, I subscribed to the Journal of Immunology. Six issues of it reached me at Brisbane in Australia on the day I boarded a ship to go North to New Guinea. In one of them I found our paper*'[66]. The photomicrograph, taken by Coons with a Leica 1 camera through a ZEISS fluorescence microscope, is the first

331 immunostaining documented and basically initiated the field of immunohistochemistry[66,67].
332 But in the early **1970s** Klaus Weber took the field a big step further, by demonstrating that an
333 organism will not just produce antibodies against actually infectious disease agents, but
334 against almost every foreign protein injected into it[62]. This realization, that antibodies can be
335 raised against pretty much any protein and then be used to label and visualize this protein in
336 other cells, formed the basis of immunofluorescence microscopy **(1974)**[62]. In order to reach
337 this breakthrough, it came in handy that Weber had previously pioneered the technique of
338 sodium dodecyl sulfate gel electrophoresis to separate and purify proteins based on their
339 molecular weight[68]. In the early 1970s, this technique allowed Weber and his colleagues to
340 obtain the pure antigens required to raise their antibodies[62]. The first antibody Weber and his
341 team raised and used as fluorescent marker was an anti-actin antibody, and the fluorescent
342 images of the actin network in chicken cells they obtained with it, served as the basis for the
343 typical textbook view of the actin cytoskeleton used for the next decades[62]. Following this
344 initial paper, the Weber lab added a string of publications lighting up the entire animal
345 cytoskeleton with antibodies against actin, tubulin, myosin and several other proteins[62,69–71]
346 **(1974-78)**. He then helped the plant field by demonstrating that *Leucojum aestivum* (summer
347 snowflake) endosperm microtubules can also be labelled with his anti-tubulin antibody,
348 providing scientists with the first view of the plant microtubule network **(1977)**[72]. Lloyd et al.
349 subsequently showed the labelling of microtubules in intact cells (*see Fig. 1 here*) **(1979)**[73].
350 The first images of the plant actin network were not obtained using antibodies, however. F-
351 Actin was first shown in the green algae *Chara* in **1980** using nitrobenzoxadiazole-labeled
352 phallacidin, while rhodamine-labelled phalloidin was used to label the actin in vascular plant
353 cells in **1985**[74,75].

354 The addition of immunofluorescence microscopy to the scientific imaging toolbox
355 represented a giant leap forward, and it set the path for the next major innovation. At this
356 stage, the capabilities of traditional light microscopes were close to being exhausted, and
357 another revolution was needed to move the field forward.

358 **A Green Fluorescent Revolution and the Visualization of Proteins (1960-1999)**

359 This revolution would eventually come with the concurrent development of the confocal laser
360 scanning microscope (CLSM) and the GREEN FLUORESCENT PROTEIN (GFP) as a
361 genetically-encoded fluorescent label. This, however, was a long process. First sketches of
362 confocal beam paths using a pinhole can be found in papers from the **1940s** and early **1950s**,
363 but the first prototype of a confocal microscope was invented, patented and built in **1955/56**

364 by Marvin Minsky[76–78]. This is somewhat peculiar, as Minsky is not known as a
365 spectroscopist, microscopist, or even biophysicist - he is a computer scientist, famous for
366 being one of the pioneers of artificial intelligence (AI) research[79]. And indeed, that is what
367 ultimately got in the way of him doing anything further with the confocal microscope
368 prototype he had built[78]. In the early 1950s, his ideas on AI were not fully matured yet, so
369 '*while those ideas were incubating I had to keep my hands busy and solving that problem of*
370 *scattered light became my conscious obsession*'[78]. And since the field of AI got going around
371 **1956**, Minsky abandoned his confocal at that point[78]. Thus, it was only in **1967** that the first
372 images were taken on a confocal microscope, more precisely on a confocal microscope using
373 a Nipkow spinning disc, named Tandem-Scanning Reflected-Light Microscope[80,81]. The
374 Nipkow disc, perforated with several small pinholes, performed a dual-function, focusing the
375 incandescent lamp illumination light beam to the layer of interest in the sample, and also
376 filtering the emitted light to get rid of any scattering out of focus light (hence the 'tandem' in
377 the name)[80,81]. Using this microscope, researchers imaged frog ganglions and noted that the
378 axons were only visible when the Nipkow disc was inserted into the microscope, thereby
379 demonstrating the ability of this technique to improve the resolution[80]. Since the image
380 quality however was not sufficiently good, they still needed to include a hand-drawn sketch,
381 explaining what was apparently visible in the image[80]. This confocal was improved in **1969**
382 with the construction of a scanning microscope, featuring a helium–neon laser as light source,
383 a moving objective lens, rather than having to move the sample, and an adjustable exit
384 aperture to act as pinhole in front of a photomultiplier detector, instead of the Nipkow disc[82].
385 The developers, Davidovits and Egger, then went on to demonstrate its ability by imaging
386 frog blood cells **(1971)**[83]. It is important to keep in mind, that these early CLSMs were still
387 used to image unstained tissue, as there were no fluorescent labels or comparable reagents.
388 The following ten years brought several more refinements and additions, such as
389 improvements in the depth of field by using confocal point scanning (the term 'confocal' is
390 mentioned here for the first time)[84–86]. From **1983** onwards, computers could be used to
391 control the microscope, and to store and process the images digitally[87,88]. And then, in **1985**,
392 Brakenhoff et al. showed that they could perform optical sectioning of samples by using a
393 computer-controlled mechanical stage that moved not just two-dimensionally, but also in the
394 third dimension, allowing them to image several layers of the same sample in confocal mode,
395 and reconstruct the three-dimensional image afterwards[89]. They used this technique to show
396 the three-dimensional arrangement of mithramycin(and therefore fluorescent)-labeled
397 chromatin in mouse nuclei – demonstrating that the CLSM had finally arrived at a state where

398 it could be used to answer a biological question **(1985)**[89,90]. When they tried to publish this
399 groundbreaking work in *Nature*, their paper, which had a title focusing on the new
400 microscopy technique, rather than the mouse, was immediately rejected on the grounds that
401 *Nature* does not publish method papers. So the authors changed the title to a less method-
402 centric '*Three-dimensional chromatin distribution in neuroblastoma nuclei shown by*
403 *confocal scanning laser microscopy*', and got the same paper published in *Nature*, since with
404 this title, it was clearly no longer a methods paper[89,91]. At the time of this publication, a
405 second paper showing a similar three-dimensional imaging approach on a CLSM was
406 published by Carlsson et al. from Stockholm University[92]. But since their work was not
407 published in *Nature*, it received less attention at the time[91]. It did however, result in the first
408 commercially available CLSM, produced by the company Sarastro[91]. This happened in
409 parallel with William Bradshaw Amos and John Graham White building their own CLSM,
410 which they also intended to commercialize[91]. In **1987**, White and Amos were the first to
411 develop a CLSM where the scanning was performed with the laser beam itself, instead of
412 moving the stage, which significantly sped up the imaging[93]. So when they submitted their
413 paper on the new CLSM to the Journal of Cell Biology, one of the editors immediately sent
414 them a note, trying to purchase the microscope[91]. The big companies, such as ZEISS and
415 Leica, were less enthusiastic, and so they eventually produced their CLSM with Bio-Rad,
416 making the Bio-Rad MRC 500 the second commercially available CLSM next to the Sarastro
417 CLSM 1000[91]. One of the first labs in the plant field to adopt the CLSM was the group of
418 Elliott Meyerowitz, who were already instrumental in pioneering *Arabidopsis thaliana* as a
419 general plant model (see also 'A Short History of *Arabidopsis thaliana* (L.) Heynh.
420 Columbia-0'[29]). In the early **1990s**, Mark Running from the Meyerowitz lab developed CLSM
421 to image *Arabidopsis* meristems, using propidium iodide as a marker for nuclei (*see Fig. 6 A*
422 *here*)[94,95]. And plant microscopists were also quick to connect the CLSM with the new field of
423 immunofluorescence microscopy. Using fluorescently-labelled tubulin, they were able to live
424 image the plant microtubule network in Tradescantia (spiderwort) on a CLSM. For this, they
425 injected fluorescein-labelled pig or sheep tubulin into plant cells, and then recorded how these
426 building blocks were incorporated into the microtubules. Furthermore, they could image time-
427 series of microtubule dynamics during mitosis and cytokinesis, and demonstrate the negative
428 effect of the herbicide oryzalin on microtubule stability (*see Fig. 1 here* & *Fig. 4 here*) **(1990-**
429 **93)**[96,97]. Also in **1993**, Grabski et al. visualized the plant endoplasmic reticulum using DiOC6,
430 and showed that it spans the entire plant cell as a net-like structure connected to the plasma
431 membrane[98]. They then used the new CLSM to already apply fluorescence recovery after

432 photobleaching (FRAP) measurements in living plant cells, demonstrating that the membrane
433 dye can actually move between cells, and that the cells' membrane systems therefore must be
434 interconnected (*see Fig. 8 here*)[98].

435 The establishment of the CLSM, in combination with fluorescent markers, was another major
436 advancement in the field of microscopy. But a second milestone had to be reached to utilize
437 its full potential, namely the engineering of GFP as a genetically-encoded reporter and
438 protein-tag. GFP was first observed in **1962**, when Osamu Shimomura and his colleagues
439 isolated bioluminescent proteins from *Aequorea* jellyfish squeezates (the result of squeezing
440 bioluminescent tissue of *Aequorea* through a handkerchief)[99]. They isolated aequorin, a
441 photoprotein that emits blue light when calcium is added. Interestingly, when stimulated in
442 intact cells, the emitted light appeared green, rather than blue[99]. Shimomura and his
443 colleagues eventually isolated the green fluorescent protein as well, and speculated that the
444 blue luminescence of aequorin could excite the green protein *in vivo*, and that this energy
445 transfer may explain the green luminescence observed in intact tissue[100]. This hypothesis was
446 confirmed in **1974**, when the calcium-triggered energy transfer between purified aequorin and
447 GFP was demonstrated *in vitro*[101]. The chromophore of GFP was then described by
448 Shimomura in **1979** (with a slight correction published in **1989)**[102,103]. At the time, the focus
449 was still quite heavily on the aequorin though, and in the early **1980s** Milton Cormier received
450 a grant from Hoffman-La Roche to clone the *aequorin* gene[104]. The pharmaceutical company
451 planned to use it as a bioluminescent marker for antibodies to use in diagnostics[104]. Cormier
452 hired Douglas Prasher for this work[104]. For the project, Prasher and his colleagues regularly
453 travelled to the island Puget Sound to go on fishing expeditions, catching fluorescent jellyfish
454 to isolate proteins, DNA and mRNA from them[104]. Using reverse transcription of the isolated
455 mRNA, Prasher constructed cDNA libraries of jellyfish to eventually isolate the specific
456 aequorin cDNA from there[104]. Since the protein structure of aequorin and GFP were already
457 partially known, Prasher could create synthetic radiolabelled antisense DNA probes to screen
458 for homologous sequences in his jellyfish libraries[104]. Using this method, Prasher and his
459 colleagues quickly progressed and were able to isolate and clone the aequorin cDNA (as well
460 as four isotypes) in **1985**[105]. Aequorin is a holoprotein, meaning that it requires conjugation of
461 a prosthetic chemical group to its apoprotein, apoaequorin, to become functional. In the case
462 of aequorin, this is a luciferin, coelenterazine[105]. Once apoaequorin and coelenterazine have
463 formed the functional aequorin, binding of two calcium ions triggers a conformational change
464 and subsequent oxidation and excitation of the coelenterazine[105–107]. As the coelenterazine
465 reverts from this excited state to its ground state, blue light is emitted[105–107]. Prasher and his

466 team were able to demonstrate and describe this mode of action when they heterologeously
467 expressed the aequorin cDNA in *E. coli* **(1985-89)**[105–107]. However, for Douglas Prasher, the
468 *GFP* gene became much more interesting[104]. Aequorin was bioluminescent, meaning light is
469 emitted by the joint action of an enzyme (in this case apoaequorin) and a light-emitting
470 molecule (coelenterazine), as well as a co-factor (calcium). GFP, however, seemed to be
471 solitarily fluorescent – able to emit light simply as a result of being excited by light of higher
472 energy. This independence of any co-factors made it a much more promising reporter in
473 Prasher's mind[104]. Following his work identifying and cloning the *aequorin* gene of *Aequorea*
474 in **1987**, Prasher received a tenure-track position at the Woods Hole Oceanographic
475 Institution, where he started to work on cloning and expressing *GFP*, trying to demonstrate its
476 usefulness as a fluorescent reporter[104]. However, not many shared his vision at the time[104]. In
477 fact, even his colleagues, like William Ward and Osamu Shimomura, reportedly doubted that
478 GFP would function as a stand-alone fluorophore[104]. And accordingly, it proved almost
479 impossible for Prasher to acquire funding for this work[104]. On top of that, Prasher felt isolated
480 and unsupported as a molecular biologist at an institution made up entirely of marine
481 biologists and ecologists, who did not appreciate his work[104]. By the early **1990s** Prasher had
482 grown so frustrated and depressed, that he decided to stop his tenure-track process at Woods
483 Hole and began to look for a new job[104]. His paper describing the successful cloning of the
484 *GFP* cDNA and gDNA was published in **1992** as his final work[108]. His last, passing-of-the-
485 torch kind of act as an academic researcher was to mail out two envelopes containing the *GFP*
486 gene, one to Martin Chalfie and one to Roger Tsien[104]. Both had read his paper and shared his
487 vision of GFP as a fluorescent protein tag[104]. Some years later, in 2008, Chalfie and Tsien,
488 together with Osamu Shimomura, were awarded the Nobel Prize in Chemistry for their work
489 on '*the discovery and development of the green fluorescent protein, GFP*'[109–111]. At the time,
490 Douglas Prasher was working as a courtesy van driver at a car dealership[104]. To acknowledge
491 Prasher's contribution, Chalfie and Tsien invited Prasher and his wife to join them at the
492 award ceremony, all costs covered[104]. Once Chalfie and Tsien had received the *GFP* gene
493 from Prasher things went fast. Chalfie and his co-workers were quickly able to express the
494 gene in *E. coli* and *Caenorhabditis elegans*, demonstrating that the protein could be produced,
495 and is indeed fluorescent without any co-factors, in both pro- and eukaryotic cells[112]. For the
496 imaging, the team used *'a variety of microscopes'*, as stated in their **1994** *Science* paper,
497 which was simply because they actually did not own a fluorescence microscope, and therefore
498 had Zeiss, Nikon and Olympus bring in demo microscopes, on which they performed their
499 experiments[112]. Chalfie also passed the *GFP* gene on to his wife, Tulle Hazelrigg, who

500 showed that it could be used in *Drosophila* as well, in a publication in *Nature* that same
501 year[113]. In his *Science* paper, Chalfie had already mentioned the suitability of *GFP* for
502 expression in *Drosophila*, a personal communication from Hazelrigg he was permitted to
503 include in exchange for, (1) freshly prepared coffee, every Saturday at 8:30 am for two
504 months, (2) preparation of a special French dinner, and (3) nightly emptying of the garbage
505 for one month[111,112]. Expression in the model yeast *Saccharomyces cerevisiae* was
506 demonstrated as well, anecdotally by the Tsien lab, and with first published images by Tim
507 Stearns **(1994/1995)**[114,115]. But Roger Tsien was primarily interested in tinkering with the
508 protein, and he quickly started publishing on new and improved variants of the fluorophore[110].
509 Single point mutations optimized its excitation properties by removing one of its two
510 excitation peaks (395/475nm), and slightly shifting the remaining main peak to 488 nm
511 **(1994/1995)**[114,116]. Furthermore, he and his team were able to create a 'cyan' variant (CFP)
512 **(1994)**[114]. Further mutations resulted in improved brightness, and the creation of a second
513 'blue' fluorophore (BFP), which the team used to demonstrate its suitability for FRET-
514 experiments (between BFP and GFP)[117]. One year later, Tsien and crystallographer James
515 Remington and their teams had determined a crystal structure for GFP and evolved the
516 'yellow' YFP **(1996)**[118]. The only 'color' that could seemingly not be engineered with GFP
517 was 'red'. But once the DsRed protein from *Discosoma* was described in **1999**, the Tsien lab
518 quickly used it to produce several red fluorophores as well, such as the monomeric mRFP and
519 the fruit collection (mCherry, tdTomato, etc.)[119–121]. An important triple-mutation not
520 engineered by the Tsien lab was added to GFP in **1996** and significantly increased the
521 brightness of the protein, resulting in the 'enhanced' EGFP[122]. Interestingly, in **2019**, the team
522 of Nathan Shaner, a student of Roger Tsien, found that the crystal jelly, *Aequorea victoria*,
523 had already naturally evolved pretty much all of the critical mutations that made the superior
524 EGFP[123], but due to its very low expression level compared to regular GFP, this variant had
525 so far been overlooked[123].

526 Thus, by 1995 GFP was successfully expressed and used in most model organisms. But
527 foreshadowing what would become a common theme for plant microscopists trying to
528 reproduce methods and techniques established in other organisms, things were a lot more
529 complicated in plants. Expression of *GFP* in plant cells only seemed to work when a virus-
530 system was used for expression of the gene, while stable transgenic *Arabidopsis* lines with
531 strong emission could not be created **(1995)**[124,125]. It was later uncovered that this was due to a
532 cryptic intron, which was spliced out in plant cells and therefore removed part of the coding
533 sequence from the *GFP* mRNA[126]. Only after codon usage optimization and removal of the

534 splice site for the cryptic intron could plant scientists finally also employ GFP as a tag for
535 their proteins as well **(1996/1997)**[127,128]. This optimized variant was first expressed in maize
536 protoplasts (*see Fig. 1 E here*), and then in stably transformed *Arabidopsis* lines (*see Fig. 4A-*
537 *H here*)[127,128]. But as always, microscopists quickly turned to their favourite structure, the
538 cytoskeleton, first showing microtubule dynamics using a new GFP-MBD (microtubule
539 binding domain) reporter for live-imaging of different cell types (*see Fig. 5 A here*), and then
540 showing a Golgi/ER/Actin co-staining (ERD2–GFP/rhodamine–phalloidin) to visualize the
541 movement of Golgi vesicles along an ER/Actin network (*see Fig. 2 e-g here*) **(1998)**[129,130].
542 The latter is a great example for the capabilities of the new techniques. The movement of
543 GFP-labelled proteins could now readily be tracked live over time[130].

544 With the advent of the CLSM and GFP a new era in microscopy began in the 1990s. The
545 constant improvements with every new generation of CLSM resulted in superior images with
546 higher resolution, and the possibility to finally label nearly every protein of choice
547 genetically, by simply fusing the *GFP* gene to the respective coding sequence, allowed
548 researchers to observe their proteins of interest in action *in vivo*. New and improved CLSM
549 techniques, and new fluorescent proteins, still many of them based on GFP, are being
550 continuously developed and released, showing that the potential of both is not yet exhausted.
551 The GFP family tree on FPbase.org is worth viewing as a very nice illustration of the wealth
552 of fluorescent proteins derived from this single protein: click here[131]. But GFP also led the
553 way toward the next big advance in microscopy, super-resolution, thanks to the '*on/off*
554 *blinking and switching behaviour*' of GFP, as observed by Roger Tsien and William Moerner
555 in **1997**[132].

556 **Plasma Membrane Nanodomains and Single Molecule Tracking (2000-today)**

557 Since the late 1980s, research on how to break the resolution limit, as defined by Ernst Abbe,
558 intensified, and in the early **2000s**, the first practical approaches were being devised and
559 tested[133–135]. Among the first super-resolution imaging techniques successfully applied to
560 resolve sub-diffraction limit structures in biological samples were stimulated emission
561 depletion (STED), photoactivated localization microscopy (PALM) and stochastic optical
562 reconstruction microscopy (STORM) **(2006)**[136–138]. The latter two of these require a blinking
563 behaviour of the fluorophores used for the imaging, as observed for GFP in 1997[132,134]. The
564 density of fluorescent labels is limiting the resolution of individual proteins, as they will
565 appear as one blur[134]. By getting them into a blinking state, only a portion of the proteins will
566 be fluorescent at a given point, thereby allowing more precise localization of their positions,

and better resolution of two or more proteins in close proximity[134]. In STED microscopy on the other hand, the transient reduction in label density is achieved by 'switching off' any fluorescent molecules in a circular area around the very center of the focal spot with a circularly polarized high-energy depletion laser[134]. This reduces fluorescence to the central spot which can have a lateral resolution of way below 100 nm[134]. For the development of such techniques, Eric Betzig, Stefan Hell and William Moerner were awarded the 2014 Nobel Prize in Chemistry[139–141]. Another super-resolution technique is structured illumination microscopy (SIM), which uses structured light patterns generated by, e.g., reflecting off a grid, to scan the focal plane multiple times[134,142]. With every scan, the pattern is shifted laterally leading to a series of images with different interference patterns[134]. The different interference patterns recorded can then be computationally reconstructed into a super-resolution image[134]. Since SIM is less invasive than the aforementioned super-resolution techniques, and can be used with conventional fluorophores, it is more compatible with live-cell imaging[134]. Sadly, SIM-developer Mats Gustafsson passed away in 2011, thereby making him ineligible for the 2014 Nobel Prize for super-resolution microscopy[143]. On top of that, the fact that SIM holds the potential for time-resolved live-cell super-resolution imaging, it is also conceivable that it will result in a Nobel Prize of its own in the future.

As always, adopting such complex new techniques to plant sciences poses a big challenge, and thus there are only few publications so far reporting on super-resolution imaging of live plant cells using these methods. This is in part because of the specialized microscopes required for these techniques. Super-resolution microscopes that allow for straight forward out-of-the-box super-resolution imaging are only now becoming more common, and the software to properly process such images is still highly complex and needs to be thoroughly understood[134,144]. However, PALM and STED have been successfully used in plants to image proteins in plasma membrane nanodomains, and to track the movement of individual proteins therein, while SIM was used to live-image the cytoskeleton (*see Fig. 2 a-d here*) **(2011-2019)**[145–148]. In the meantime, plant microscopists have taken advantage of the range of near super-resolution techniques, which can be performed on regular confocal microscopes with additional hardware components and better deconvolution software, such as total internal reflection fluorescence (TIRF) microscopy, the ZEISS AiryScan setup, or fluctuation-based super resolution microscopy techniques, such as super-resolution radial fluctuations (SRRF) imaging[149–153]. The AiryScan and single-molecule TIRF have also been successfully used in plants to study single proteins in plasma membrane nanodomains (*see Video 2 here*), while SRRF is used for less mobile structures like cell wall components (*see Fig. 2 E here*) **(2011-**

601 **2021)**[149]. And, of course, these techniques have also been used on the cytoskeleton (*Fig. 1 A-*
602 *F here*)[148,150]. Beyond this, plant microscopists have achieved close to super-resolution images
603 using spinning-disc confocal microscopes equipped with super-fast high-resolution cameras.
604 Using such a microscope the group of Akihiko Nakano was able to simultaneously live-image
605 the directed trafficking and sorting of several distinct proteins, labelled with different
606 fluorophores, within the trans-Golgi network **(2021)**[154]. That same year, and again using such
607 a spinning-disc confocal, the rearrangement of individual microtubules into thick, regularly
608 spaced bundles, required for secondary cell wall pattern formation in single cells *in planta*
609 was achieved (*compare the 2021 live-video S2 here to the 1955 phase-contrast image of the*
610 *secondary cell wall in Fig. 7 here*)[44,155].

611 Overall, super-resolution-ready microscopes are now part of the product range of all the big
612 microscope suppliers, such as ZEISS, Nikon, Leica or Andor, and some small manufacturers
613 have also emerged specialized on specific super-resolution techniques. Among them, Nobel
614 laureate Stefan Hell is one of the founders of Abberior Instruments, which focuses on the
615 STED technique developed by Hell. One of their specialized STED microscopes has recently
616 been employed to image the distinct localization of two chromosomal proteins in *Arabidopsis*
617 at super-resolution (*see Fig. 1 A,B here*) **(2021)**[156]. Accordingly, it appears that the dawn of
618 super-resolution has now also arrived for plant microscopists.

619 **Vertical-Stages, Light-Sheets and Open Science (2000-today)**

620 At this stage, with custom-built or customized microscopes becoming more common, plant
621 microscopists could also finally tackle a problem unique to the field: tilting the imaging stage
622 into a vertical position. As plants grow along the gravitational vector – roots with, shoots
623 against it – long-term live-imaging of developmental processes should ideally be performed
624 with the plants positioned vertically. Use of a vertical-stage microscope was first reported in a
625 **2009** paper studying the response of a root growing against a physical barrier[157].
626 Subsequently, it was used to study the interplay between gravity perception and hormone
627 signalling in the root **(2017/18)**[158,159].

628 And finally, the early 2000s also brought us the light sheet fluorescence microscope
629 (LSFM)[160,161]. In a LSFM the excitation light is focused only along one axis to create a thin
630 planar sheet of light, instead of a spot[161]. This planar sheet of light then illuminates a complete
631 slice of a sample, which is imaged at once through an objective arranged at a 90 degree angle
632 to the light sheet[161]. By moving the sheet through the sample slice by slice along the Z axis,

633 three-dimensional images can be quickly obtained[161]. The design and implementation of the
634 first LSFM was published by Richard Zsigmondy in **1909**, and featured an illumination light
635 path that converted polarized sunlight into a light sheet by simply channelling it through a thin
636 slit[162]. Using this ultramicroscope, as he called it, he was able to image gold particles in a
637 colloidal gold solution, which could not be imaged with the standard microscopes at the
638 time[162]. For this work he was award the Nobel Prize in chemistry in **1925**[163]. Following this
639 breakthrough however, things got rather quiet around light sheet microscopy for nearly a
640 century. A similar technique was published in **1993** as orthogonal-plane fluorescence optical
641 sectioning, but like Zsigmondy's ultramicroscope, it did not catch on[164]. Things only changed
642 in **2004**, when the lab of Ernst Stelzer published its selective plane illumination microscope
643 (SPIM)[160]. Stelzer subsequently collaborated with plant microscopist Alexis Maizel to adapt
644 the SPIM for studies with plants, using it first to create high-resolution three-dimensional
645 time-series of growing roots and lateral roots (*see Video S1 here*) **(2011)**[165]. The SPIM design
646 was eventually commercialize by the EMBL-spin out company Luxendo, who's LSFM have
647 also been used successfully in plants **(2020)**[166].

648 The SPIM also stands as an example for the growing open science movement[167]. In **2013**, Jan
649 Huisken, first author of the 2004 SPIM paper from the Stelzer lab, teamed up with Pavel
650 Tomancak to create the OpenSPIM platform (http://openspim.org/), making everything
651 needed to custom-build one's own SPIM openly available to the community[167]. And, in
652 similar fashion, this community-based thinking of the open science movement has benefitted
653 microscopists in many other ways. Another prime example is the image-analysis software
654 Fiji[168]. Based on the National Institutes of Health's ImageJ, Fiji is an open-source,
655 customizable, all-in-one image analysis program, which nowadays is indispensable for
656 microscopists from all fields (the paper has so far been cited over 25000 times, despite many
657 authors neglecting to cite it in the methods section of their papers) **(2012)**[168,169]. ImageJ/Fiji
658 allows users to write and incorporate new tools and plug-ins, and the SRRF analysis open
659 source toolkit mentioned before is an example of one such plug-in[170]. MorphoGraphX is an
660 open-source 3D image processing/analysis program, which not only allows for three-
661 dimensional image-reconstruction, but also cell segmentation and cell lineage tracing, and
662 carries the additional advantage to plant microscopists that it was developed with plant
663 scientists **(2015)**[171]. And finally, with the ever-increasing selection of fluorescent proteins
664 available to microscopists, Talley Lambert has recently created the community-editable
665 FPbase database (https://www.fpbase.org), an invaluable resource of all information available
666 for any fluorescent protein[131].

In the coming years, it can be expected that super-resolution microscopy will fully enter the plant field, as more groups specialize on the adoption and establishment of these techniques, and more companies produce custom-made microscopes that make it easier to apply them straight out-of-the-box. Plant optogenetics is another emerging research area with increasing use and applicability of microscopy methods, which will become increasingly important in the coming years to engineer and control pathways in plants[172,173]. With an ever-growing open-science movement, improved data/image-analysis tools, programs and databases are constantly being developed and made publicly available, making every step from image acquisition to publication easier. Accordingly, we can expect many more beautiful (and informative) images of plants at an ever-increasing resolution in the years to come.

Further Reading:

- A Short History of Plant Science
  Chapter 1: A Short History of Arabidopsis thaliana (L.) Heynh. Columbia-0[29]
  Chapter 2: A Short History of the CaMV 35S Promoter[59]
  Chapter 3: A Short History of Plant Transformation[60]
  Chapter 4: A Short History of Vernalization[174]
- Nehemiah Grew - The anatomy of plants - with an idea of a philosophical history of plants, and several other lectures, read before the Royal Society[1]
- Howard Gest - The discovery of microorganisms by Robert Hooke and Antoni van Leeuwenhoek, Fellows of The Royal Society[4]
- Harald Volkmann - Ernst Abbe and his work[32]
- W. B. Amos & J. G. White - How the Confocal Laser Scanning Microscope entered Biological Research[91]
- Yudhijit Bhattacharjee - How Bad Luck & Bad Networking Cost Douglas Prasher a Nobel Prize[104]

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