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1 **Clearing plant tissues: advances and promising practices**

2

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11

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32 **Abstract**

33 The study of the organ structure of plants and understanding their physiological complexity  
34 requires 3D imaging with subcellular resolution. Most plant organs are highly opaque to  
35 light, therefore their study under optical sectioning microscopes is difficult. In animals, many  
36 protocols have been developed to make organs transparent to light using clearing protocols  
37 (CPs). By contrast, clearing of plant tissues is a challenge due to the presence of fibres and  
38 pigments. In this review, we describe how progress of plant CPs was achieved throughout  
39 the past twenty years by an adapted taxonomy, implemented by physical and optical  
40 parameters affecting tissue properties. We also discuss successful approaches combining CPs  
41 and promising microscopy methods and their future applications in plant science research.

42

43 **Aim to observe in depth plant tissues**

44 Since Hooke's first observation and description of cork cuts in the middle of the 17<sup>th</sup>  
45 century, plant science research has remained closely related to advances in optical  
46 microscopy. More specifically, the use of fluorescence labeling and optical sectioning has  
47 drastically improved the understanding of tissue micromorphology [1], cell development [2],  
48 protein localization and plant-microbe interactions [3]. These microscopy approaches are,  
49 however, limited to approximately 30  $\mu\text{m}$  depth because of the opacity and auto-  
50 fluorescence of plant tissues. Opacity is mainly due to (i) light absorption by pigments, and  
51 (ii) light scattering by the wide range of **Refractive Indices (RI, see Glossary)** of cell  
52 components (Figure 1). Moreover, plants contain a lot of light-sensitive pigments and  
53 aromatic compounds that animal tissues do not have. These compounds can react to laser  
54 excitation from microscope and generate a significant noise impairing the fluorescent signal  
55 of interest. Two approaches are available to overcome limitations related to depth and  
56 chemical composition. The usual approach consists in mechanical sectioning. This type of  
57 sample preparation is time consuming, section borders can be damaged (causing a loss of  
58 cell content) and 3D reconstruction can be approximate, leading to a loss of information [4].  
59 Another approach consists in clearing sample by chemical treatments. Clearing protocols  
60 (CPs) make sample transparent to light by (i) removing light-active compounds, and (ii)  
61 homogenizing RI inside plant tissue (Box 1). This process allows the acquisition of a  
62 fluorescent signal through a thick sample. Over the past twenty years, many CPs have been

63 developed for animal tissues (*e.g.* zebrafish, mouse), mainly in neuroscience to study brain  
64 tissues; followed by application to other organs, including bones, up to the whole body [5].

65 Here, we review all CPs that have been applied and adapted from animal-based  
66 observation or specifically developed for plant tissues to gain new insights into plant  
67 physiology and develop prospects in the field. First, we apply the CPs classification in force  
68 based on organic solvents and aqueous solutions with passive immersion, protein  
69 hyperhydration and hydrogel embedding. Next, we discuss briefly chemical and physical  
70 impacts of CPs on tissue properties. Finally, we present successful CPs that have been  
71 applied to animals tissues and combined with recently developed microscopy techniques for  
72 being further transferred and optimized on plant organs. We aim to provide helpful guidance  
73 for scientists to select the most appropriate CP among existing ones, or to develop their own  
74 clearing approach, depending on plant tissue and scientific issue.

75

## 76 **A glimpse of the historical record of clearing reagents**

77 The work of Spalteholz in 1911 [6] was one of the first record to have used clearing  
78 agents on human organs. In plant research, lactic acid [7] and chloral hydrate [8,9] initially  
79 used as a mounting and preserving medium in Hoyer's solution [10], were two commonly  
80 used clearing agents. Their ability to clear tissues relies on their RI of 1.43, very close to plant  
81 cell wall RI (1.42). Chloral hydrate has been used in many CPs on a wide range of plants,  
82 especially on *Arabidopsis thaliana* (*A. thaliana*), to clear various organs (*e.g.* the phloem [11],  
83 leaves [12], flower organs and siliques [13]), and in combination with diverse fixation  
84 techniques and staining agents (*e.g.* acid fuchsin [8], aniline blue [14], GUS [11]). In  
85 fluorescence-based microscopy, chloral hydrate has been combined with the fluorescence of  
86 modified **Pseudo-Schiff reagents** - Propidium Iodide (mPS-PI) to visualize the plant cell wall  
87 [12]. Nevertheless, chloral hydrate treatment leads to endogenous fluorescence quenching,  
88 that made it not very popular [15] in addition to its anaesthetic effect.

89 The need to observe plant tissues in depth has led to the independent development of  
90 many CPs depending on the sample (*e.g.* reproductive organs [16]), structure of interest (*e.g.*  
91 lignin [17]) and timescale (*e.g.* one-step clearing [18]). Gardner summarized CP steps such as  
92 fixation, alkali dissolution, post-alkali treatment, depigmentation, crystal removal, high-RI  
93 solution and staining [19]. Until then, microscopes used for such observations had not  
94 reached subcellular-level resolution. Advances in optical sectioning microscopy (confocal,

95 two-photon, light sheet microscopes) and increasing contrast and resolution paved the way  
96 for many CPs that allowed **Fluorescent Probes (FP)** imaging.

97

### 98 **Clearing protocols for plant tissues**

99 Recent animal CPs are usually classified into two main categories based on  
100 physicochemical properties of clearing agents: (i) organic solvents and (ii) aqueous solutions  
101 [20]. What about plant CPs for FP imaging?

#### 102 *Solvent-based clearing*

103 The pioneer CP [6] followed a procedure that was standardized and described in animals:  
104 dehydration, lipid solvation and incubation in a high RI solution [21]. Few recent methods  
105 have been described in animal research, especially for brain tissue [22], but the literature  
106 about plant research is poorly documented.

107 Littlejohn et al. used perfluorocarbons as a mounting medium to observe *A. thaliana*  
108 leaves by confocal microscopy. Although the authors never employed the term “clearing” in  
109 the development of this protocol over the years [23,24], their approach relied on the  
110 homogenization of the sample RI (Box 1). The RI of perfluoroperhydrophenanthrene (PP11,  
111 1.334) is very close to the RI of cytoplasm (1.35 – 1.39), but far from the RI of air (1.00).  
112 Littlejohn et al. had to deal with the spongy mesophyll of *A. thaliana* leaves, meaning that  
113 they needed an agent able to replace air in leaf cavities and with a RI close to that of  
114 cytoplasm in order to ensure the path of light through leaf tissue. Using this reagent, they  
115 successfully cleared non-fixed leaves in 10 min and succeeded in observing a GFP signal at a  
116 depth of 75  $\mu\text{m}$ [24]. This low RI solution, however, has never been applied to any other plant  
117 organ [25].

118 The story of Visikol™ started in 2013, when Villani and colleagues [26] published the  
119 efficiency of this new clearing agent formulation (RI = 1.445) designed to replace chloral  
120 hydrate, and successfully tested on small plant samples (fresh or dried). The clearing step  
121 consisted in heating (60-80°C) sample in Visikol™ on a slide for less than one minute.  
122 Visikol™ was compared with chloral hydrate and potassium hydroxide, using different  
123 mounting media, under confocal and airyscan microscopes [27]. They treated leaves for 3 to  
124 24 hrs with Visikol™ at 40°C to observe cuticle stained by PI. Visikol™ won the game thanks

125 to its nontoxicity and affordability. Later on, *A. thaliana* root tips were cleared using  
126 Visikol™ and successfully stained by GUS histochemical labeling (CyclinB1;1-GUS, [28]).

127 The benzyl benzoate/benzyl alcohol mixture (1:2 (v:v) **BABB**; RI = 1.559) – a widely used  
128 clearing agent in animal CPs – has been little used in plant CPs. BABB was used to initially  
129 image *A. thaliana* under Optical Projection Tomography (OPT) [29] before the authors  
130 improve their instrument to perform a macro-OPT (M-OPT, see section 2) to image *A.*  
131 *thaliana* veins by GUS staining in 3D in an entire plant [30]. Plants were fixed in acetone for  
132 20 min, GUS-stained, dehydrated (methanol, 1 week), cleared and mounted in BABB. Galls  
133 caused by nematodes were studied on *A. thaliana* roots [31]. They performed  
134 glutaraldehyde fixation (overnight), ethanol dehydration (overnight), clearing and mounting  
135 in BABB to visualize a complete gall structure with a nematode in 3D only by recording the  
136 autofluorescence signal on a laser scanning confocal microscope. In fact, both protocols  
137 induced severe fluorescence quenching and BABB potentially induced rapid peroxide  
138 contamination that damaged FP [32]. In the latest version of the protocol – FluoClearBABB –,  
139 the dehydration step is modified (*tert*-butanol, 7 days) and a basic pH (9.5) is maintained  
140 [33]. This CP allowed the authors to record enhanced Green Fluorescent Protein (eGFP)  
141 signals in an entire mice brain under a home-built light sheet fluorescent microscope.  
142 Unfortunately, this CP is not referenced yet in plant tissue literature.

143 Methyl salicylate, another widely used clearing agent (RI 1.53) [6,34], has recently  
144 been combined with FP imaging. On *Zea mays* ovules, Kalinowska and colleagues performed  
145 a formalin-acetic acid-alcohol fixation (FAA, various concentrations and time depending on  
146 organs) and made Feulgen staining (hours). Then, they made a gradual ethanol dehydration  
147 (hours) before clearing in methyl salicylate (hours) [35]. They observed on a confocal  
148 microscope a good level of transparency on these very dense organs at different stage of  
149 development, but no acquisition in z was reported. González-Gutiérrez and colleagues, fixed  
150 Crassinucellate ovules (formaldehyde (FA)), permeabilized membranes and cuticles  
151 (acetone), stained tissues by rhodamine-phalloidin (F-actin marker) and Hoescht (nuclei  
152 marker), dehydrated (isopropanol) and cleared (methyl salicylate) tissues. In their study,  
153 authors precise that this CP can be done in 48 hrs [36]. Under a confocal microscope, they  
154 successfully imaged actin network of entire ovule but once again, no acquisition in z was  
155 reported. This year, He and colleague [37] developed a new CP for crop roots based on

156 methyl salicylate. They proceed to a fixation step (15.5 hrs) with paraformaldehyde (PFA)  
157 and dimethylsulfoxide (DMSO), an RNase treatment (12hrs), a PI staining (overnight), a  
158 dehydration step in ethanol (4 hrs) and at least clearing by methyl salicylate immersion  
159 (overnight). Under a confocal microscope, they successfully imaged PI fluorescent signal on  
160 entire rice root tips thickness, but endogenous GFP signal were lost.

161 Due to dehydration step, solvent based CPs are known to induce shrinkage on soft  
162 tissues [5]. In the literature cited above, this phenomenon is not reported except for thin  
163 root tips of *A. thaliana* [37]. It can be explained by the presence of cell walls in plant tissue,  
164 but more studies are needed to confirm the absence of shrinkage during clearing of whole  
165 organs.

166

#### 167 *Aqueous-based clearing*

168 Hydrophilic CPs involve three techniques: passive immersion, protein hyperhydration  
169 and hydrogel embedding. A wide range of aqueous-based protocols developed on animal  
170 tissue have been adapted to plants. Aqueous-based CPs are compatible with FPs but are  
171 relatively time-consuming compared to solvent-based CPs [5].

172 In simple immersion, the lipid content of the sample is preserved. This CP consists in  
173 matching the RI of lipid tissue (that is greater than 1.45 in animals) thanks to aqueous  
174 solutions with a high RI solution [21].

175 The See Deep Brain (SeeDB) is a mixture of fructose (80.2% w/w) and 0.5%  $\alpha$ -  
176 thioglycerol, and a known efficient clearing agent on brain tissue [38]. It was tested on  
177 various plant leaves, including tree leaves [39]. One-cm diameter leaf discs were drop fixed  
178 in FAA, cleared in SeeDB by immersion (1-3 days), and stained with PI and calcofluor white.  
179 SeeDB was not as efficient as other tested chemical reagents such as 1% sodium  
180 hypochlorite (NaClO) on leaves. This publication is liable to criticism because (i) the chemical  
181 reaction of NaClO into the tissue is not detailed and (ii) even if NaClO is not recording as a  
182 clearing agent in animals, it has already been applied on plant leaves [40].

183 2,2'-thiodiethanol (TDE) was first used as a mounting medium [41]. Its high RI is  
184 proportional to its concentration (1.374 - 20%; 1.515 - 97%) and makes it an effective, user-  
185 friendly clearing agent, suitable for various microscopy systems and samples [42,43]. In plant  
186 tissues, TDE was used in a CP called Transparent plant Organ MEthod for Imaging (TOMEI) to

187 clear *A. thaliana* and *Oryza sativa* organs [44]. Without sectioning and with a fixation step  
188 based on acetic acid:ethanol (1:3, 1-2hrs, TOMEI-I) or PFA (4%, 1hr, TOMEI-II), they analyzed  
189 cell structures at a depth of 200  $\mu\text{m}$  in a few hours. In *A. thaliana*, fixation with 4% PFA  
190 turned out to be a necessary step before using TDE to avoid tissue disruption and FP  
191 quenching [45]. Whatever the fixation step, a high concentration of TDE (95% - 100%) can  
192 weaken the fluorescent signal of some endogenous FP (*e.g.* Venus-YFP and eGFP are more  
193 sensitive to quenching than mCherry and Citrine-YFP), and lead to a loss of tissue integrity.  
194 These authors also determined that a 50% - 70% TDE solution was necessary and sufficient  
195 to clear challenging plant tissues while preserving the structure and the fluorescent signal.  
196 Nevertheless, later comparative studies revealed that TOMEI doesn't remove all pigments  
197 and can decrease FP intensity [45]. To overcome these limitations, an update of this CP,  
198 called iTOMEI (improved TOMEI) was published in 2021 [46]. They convey a large screening  
199 of chemical treatments for each CP steps based on (i) preservation of GFP signal, (ii)  
200 enhancement of GFP signal and (iii) clearing efficiency. After comparison, they proposed a  
201 fixation step with 1% FA in PBS buffer, a decolourization step with 20% caprylyl sulfobetaine  
202 (a detergent) in sodium phosphate buffer at pH 8.0 and a clearing step with 70.4% iohexol  
203 instead of TDE. They successfully imaged entire *A. thaliana* seedlings, organs of *O. sativa* and  
204 *Marchantia polymorpha* at a depth of 80 $\mu\text{m}$  and 200 $\mu\text{m}$  respectively, under confocal or two  
205 photons microscopes. RI of the solutions was not provided, but iohexol solution seems to be  
206 prepared in the same buffer and at the same concentration as in SeeDB2 CP (an upgrade of  
207 SeeDB CP) [47]. In iTOMEI, detergent is used to improve chlorophyll elution, so iTOMEI is not  
208 just a "simple immersion" CP as TOMEI is. Nonetheless, as SeeDB2 required saponin as a  
209 detergent and is still classified into "simple immersion" category in animal CP taxonomy [5],  
210 we chose to keep iTOMEI in this category (Figure 2). Concerning caprylyl sulfobetaine, it is  
211 referenced as a zwitterionic detergent solvating oleosins. Hence, we can easily ask for the  
212 impact of iTOMEI on protein and lipid contents of cleared sample.

213 Another approach consists in decreasing and then homogenizing the RI of a sample in  
214 two steps: (i) removal of lipid content (delipidation) and (ii) hyperhydration of proteins. In  
215 order to maintain an aqueous-based environment to support FP activity, delipidation is  
216 performed using a detergent. This step is longer compared to delipidation with an organic  
217 solvent (weeks to months *versus* hours) [21]. Following delipidation using Triton X and



218 hyperhydration using urea, the Scale technique decreased the RI of mouse brain tissue to  
219 1.38 [48]. In plants, delipidation using Triton X in glycerol was combined with urea on leaves  
220 (*Zea mays*, *Medicago truncatula*, *Nicotiana benthamiana*, *Pisum sativum*) and nodules of  
221 leguminous plants (*P. sativum*) [49]. Fixation was achieved using PFA or glutaraldehyde. The  
222 staining step was adapted according to plant tissue (Table 1). Warner and colleagues [49]  
223 succeeded in imaging a fluorescent signal at more than 350 $\mu$ m depth on intact nodules and  
224 immunolabeling sections of cleared nodules (by adding an enzymatic digestion step to the  
225 CP). Recently, this CP has been used to clear *Lotus japonicus* roots and nodules to visualize  
226 auxin and cytokin dynamics and cell division patterns with cellular resolution during  
227 nodulation thanks to markers optimized for fluorescent and bright field microscopy [50].  
228 Following Warner's pattern of combining polyhydric alcohol, detergent and urea. Kurihara et  
229 al. [15] carried out a large chemical screening. It was based on (i) the clearing efficiency of  
230 different compounds from the literature (especially efficient to remove chlorophyll  
231 autofluorescence), (ii) the suitability of this compound with recombinant Venus  
232 fluorescence, and (iii) the clearing efficiency of mixtures containing selected compounds and  
233 detergents. They concluded that ClearSee (xylitol/sodium deoxycholate/urea) has a good  
234 clearing efficacy, and was suited for a large range of endogenous FPs (*e.g.* mTFP1, sGFP,  
235 mClover, Venus, mCitrine, tdTomato, mApple), numerous organs (leaves, roots, seedlings,  
236 pistils, stems and protonemata) and plant species (*A. thaliana*, *Physcomitrella patens*). Since  
237 then, ClearSee has been applied to a wide range of plant species and organs [51–55]. Nagaki  
238 and colleagues [56] optimized the initial ClearSee method for immunohistochemistry  
239 (namely ePro-ClearSee). They added an enzymatic digestion step by cellulase and  
240 pectolyase, and a 2-propanol treatment step (permeabilization) before clearing (timescale:  
241 10 days to 3 weeks). Using confocal microscope with or without airyscan detector, they  
242 acquired a fluorescent signal from modified immunolabeled histones in leaves and roots of  
243 various plant species. In the latest update of this protocol, named ClearSeeAlpha, a  
244 reductant (sodium sulfite 50 mM) was added to avoid brown pigmentation due to  
245 polyphenol oxidation [57]. These authors successfully cleared brown-pigmentation-sensitive  
246 leaves and floral organs of various plant species. Moreover, using a multiphoton confocal  
247 microscope, they demonstrated the suitability of this protocol with endogenous FPs. By  
248 combining a depigmentation step previously developed on *Xenopus* [58], an enzymatic  
249 digestion based on ePro-ClearSee and a clearing step with ClearSeeAlpha, the Clearing

250 Tannic Roots (CTR) protocol enabled acquisition of a PI signal on entire *Vitis vinifera* root  
251 thickness (250 $\mu$ m in z) under a confocal microscope [59]. Authors noticed that the time  
252 required has to be adapted according to root age : the older the tissue, the longer each step  
253 takes.

254 To avoid potential removal of key structural proteins by solvents and concentrated  
255 detergents, Chung and colleagues [60] introduced another aqueous CP, consisting in fixing  
256 and embedding the sample in a polyacrylamide hydrogel. Combined with a low-  
257 concentrated Sodium Dodecyl Sulfate solution (SDS 8%) and electrophoresis, the **CLARITY**  
258 method removed lipids and other light-active compounds. In plant tissues, Palmer and  
259 colleagues [61] added an enzymatic degradation step to the passive CLARITY protocol to  
260 clear *Nicotiana tabacum* leaves. They called their method Plant-Enzyme-Assisted CLARITY  
261 (PEA-CLARITY). The enzyme cocktail (cellulase, xylanase, arabinofuranosidase, pectate lyase  
262 and  $\alpha$ -amylase) is directed against cell wall to enhance its permeability to fluorescent dyes,  
263 especially antibodies, for better immunolabeling efficiency. The hydrogel embedding  
264 medium prevents tissue collapse by providing a supportive network. Finally, RI  
265 homogenization is done by immersion in a high RI solution like TDE [62], and can record the  
266 fluorescent immunolabeled signal of an entire tobacco leaf (200  $\mu$ m depth)(Table 1).

267 Aqueous based CPs are well described to induce swelling of soft samples. While  
268 expansion phenomenon is used in animal cleared organ to gain in resolution [63], its use in  
269 plant organs is still not reported, probably because of plant cell wall. A large part of aqueous  
270 based-CPs, however, was developed to modify and elute cell components (*e.g.* complex  
271 sugars, proteins and lipids). According to the treatment strength, a tissue can loss its  
272 integrity and become weaker [62].

273

#### 274 **Optical clearing methods serving optical sectioning techniques**

275 Until the middle of the twentieth century, in-depth observation of tissues was still  
276 limited by the efficiency of fluorescence microscopes. When observing a thick sample on a  
277 standard fluorescence microscope, only the surface could be imaged. In depth, the signal in  
278 focus was blurred by the out-of-focus fluorescent signal of the rest of the sample. The  
279 challenge of optical sectioning was to remove this out-of-focus background. The

280 development of computer-aided optical sectioning microscopy paved the way for 3D  
281 microscopy and promoted clearing agent applications [32].

282 Optical sectioning consists in selecting a specific  $(x, y)$  plane and in acquiring this plane at  
283 multiple depths. The acquired images are called z-stacks; the lower the interval between z-  
284 stacks, the higher z resolution. Optical sectioning developed along with the use of confocal  
285 microscopy. This technique is based on wide-field illumination; thanks to the pinhole, only  
286 photons emitted from the focal plan are collected, leading to removal of the out-of-focus  
287 background. Photon dispersion, however, increases with depth of acquisition [64]. This is  
288 one of the reasons why confocal microscopy of thick samples is highly dependent on CP.  
289 Another way of avoiding the out-of-focus background is to avoid generating it through  
290 specific illumination. Two-photon and light sheet illumination were designed to answer this  
291 question. Compared to confocal microscopy, imaging process is faster and less phototoxic  
292 [65] [66]. **Light Sheet Fluorescence Microscopy (LSFM)** has many applications combined  
293 with clearing agents, hence different names across publications (Box 2). Silvestri and  
294 colleagues [20] present a complete overview of CPs adapted for each of the three types of  
295 microscopy techniques (confocal, two-photon and LSFM) according to their physical  
296 principles.

297 Moreover, another factor impacting depth imaging are spherical aberrations [67]. These  
298 aberrations are evaluated by the **Point Spread Function (PSF)** that described the image of a  
299 single point through an optical system, called convolution. This function determined the  
300 resolution of an optical system (each microscopy type has its own shape type of PSF) and PSF  
301 depends on the RI of the entire imaging system. Even if CP aim to homogenise RI between  
302 tissue and embedding medium, mismatch between RI of the objective medium lens and  
303 clearing solution generate aberrations, leading to an extended PSF and a decrease in spatial  
304 resolution [67]. To deal with this problem, microscope manufacturer could propose either  
305 objectives with correction RI collar or specific objective matching clearing solution RI [68].  
306 Moreover, two numerical criteria can be calculated to predict the loss of image quality and  
307 the best objective to use according to the clearing agent [69]. A post computational  
308 treatment, called deconvolution, can also be applied to lower aberrations. This operation  
309 reassigns the aberrated light to its original source thanks to the theoretical PSF of the optical  
310 system (*e.g.* Huygens software).

311 Even if LSM is the most widely used microscope technique, cleared tissue can be  
312 imaged with a wide range of microscopes.

313 Optical projection tomography (OPT) is an adaption of tomography from X-ray imaging to  
314 optical systems [70]. In this system, the entire sample is placed in a cylinder and rotated  
315 during acquisition. Light transmission images – called projections or tomographic sections –  
316 are collected in different directions. Then, 3D reconstruction based on that projection is  
317 done using an algorithm [71]. The technique is compatible with fluorescence, and the system  
318 is known as emission OPT [72]. OPT systems are highly sensitive to tissue scattering, so that  
319 their optimization is closely related to the development of clearing agents. Lee and  
320 colleagues [29] first observed BABB-cleared *A. thaliana* various organs under an OPT system  
321 but they could not go beyond 15 mm depth. Consequently, they published an article about a  
322 Macro-OPT (M-OPT) system in 2016 [30] that allowed 3D imaging of very large specimens  
323 (up to 60 mm tall and 45 mm deep) and could be used with any clearing agent (resolution  
324 range : from 6.5 to 62.5  $\mu\text{m}/\text{voxel}$ ). Here, M-OPT is not reported to image FP signal in  
325 cleared plant tissue. Nevertheless, as OPT is suitable with fluorescent imaging and, new user-  
326 friendly hardware and software are available [73], M-OPT promises new insight deep volume  
327 imaging in cleared plant tissue.

328 Other devices have been used with clearing agents, but still not applied to plant tissue.

329 **Stimulated Raman Scattering microscopy (micro-SRS)** is a recent microscopy technique  
330 based on the combination of optical microscopy and Raman spectroscopy. This instrument  
331 can map chemical bonds in 3D, with subcellular resolution, but laser emission is sensitive to  
332 scattering, and depth imaging is limited to 100  $\mu\text{m}$  [74]. To solve this problem, CPs suitable  
333 with Raman spectroscopy were designed to achieve lipid and protein mapping of brain tissue  
334 across 1 mm depth [74] and to image collagen and hydroxyapatite during skull clearing  
335 process [75]. Micro-SRS was first used on *A. thaliana* leaves [76], and then to study the  
336 chemical composition of cuticular waxes and cell walls, and to investigate agrochemicals in  
337 maize and cotton leaves [77]. The authors, however, did not mention any acquisition deep  
338 into the tissue, and micro-SRS applied to cleared plant samples is still lacking.

339 Hyperspectral dark field microscopy images nanoparticles based on their refractive  
340 spectral signature. In this way, it is possible to label many biological structures of interest  
341 (*e.g.*, mRNA, membrane proteins) with plasmonic nanoparticles (metals). Although contrast

342 in dark field microscopy is generated by tissue scattering, the plasmonic signal is impaired by  
343 this background [78]. By clearing human cells (*in vitro* lines), the signal-to-noise ratio of gold  
344 nanoparticles is 5 to 6 times greater, so that monomeric and oligomeric forms of  
345 transmembrane receptors can be distinguished and mRNA can be detected at single copy-  
346 resolution [78]. At tissue scale, clearing human skin samples allowed 2D and 3D mapping of  
347 plasmonic nanoparticles signals, and improved the signal to noise ratio [79]. Many studies  
348 exist on tracing metal particles in animal tissues *via* this microscopy technique, but clearing  
349 process are not routinely applied [80]. Concerning plant tissues, *A. thaliana* roots and  
350 various legume leaves have been analyzed under a hyperspectral dark field microscope to  
351 image gold and iron particles, respectively, after exposure during germination [81,82]. Once  
352 again, no CP has been reported for these tissues.

353 STimulated Emission Depletion (STED) is a super-resolution microscopy technique based  
354 on the destruction of the fluorescence emission signal around the initial point of laser  
355 excitation. Many parameters can be modulated on a STED microscope, including  
356 implantation with other optical techniques, to overcome the specific constraints imposed by  
357 sample specificities and image them in 3D [83]. Like all optical fluorescence microscopes,  
358 however, it is highly sensitive to light scattering and spherical aberrations and can be easily  
359 combined with a CP when using fixed tissue. By adapting the SeeDB-based protocol to super  
360 resolution microscopy (SeeDB2), FPs signal was acquired through a commercial STED  
361 microscope (chemical fluorophores and fluorescent proteins) in mouse brain down to 100  
362  $\mu\text{m}$  depth, with 50 nm lateral resolution [47]. Angibaud and colleagues [84] cleared mouse  
363 brain using **CMF3** (RI = 1.518) and imaged it by a STED immunofluorescent signal at a depth  
364 of 40  $\mu\text{m}$ . As Komis and colleagues mentioned [85] in their review, observation of plant  
365 tissues by STED techniques are very challenging . One of their outstanding questions – how  
366 to overcome the light-scattering properties of bulk plant samples with greatly varying  
367 refractive indices in the axial direction – could probably be answered by an adapted CP [86].

368

### 369 **Concluding remarks**

370 CPs have already provided new insights into plant tissues and chlorophyllous organs. But,  
371 no single plant CP can suit all imaging projects because of plant tissues and organs diversity.  
372 Further optimization on organs containing certain light active compounds (*e.g.* phenols) is

373 needed [87]. Therefore, future CPs will have to be based on systematic chemical screening  
374 because all organs vary in their lipid, protein and sugar compositions. Moreover,  
375 comparative studies of various CPs applied on the same organ are necessary to evaluate the  
376 most suited one with an emphasis on CPs compatible with specific fluorescent dyes (*e.g.*  
377 immunofluorescence [88]). Compared to publications on animal tissues [89], the field of CPs  
378 on plant tissue is still in its early stages, but it is promising approach to unravel anatomical  
379 and physiological insights in plant-microbes interactions (see Outstanding Questions). CPs  
380 need to be seen in the context of studying plant physiology at different scales, from the  
381 entire plant down to the organ level and from the cell to the protein network.

382

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604

## Glossary

605 **Benzyl Benzoate/Benzyl Alcohol (BABB):** mixture is the precursor of all organic solvent-  
606 based protocol in animals [5,90].

607 **CLARITY:** a protocol designed to render a tissue optically transparent and permeable to  
608 macromolecules while keeping its shape using hydrogel embedding [54].

609 **CMF3:** a commercial mounting medium with a high RI (1.518) used as clearing agent and  
610 suitable with fluorescent immunolabelling [74].

611 **Fluorescent Probe (FP):** refers to all fluorescent makers (exo- and endo-genous). Here, in the  
612 review, “endogenous FP” specifically refers to encoded fluorescent proteins.

613 **pseudo-Schiff reagents:** Schiff reagents are histochemical reagents used to detect aldehydes  
614 functions in a tissue after a reaction with a dye containing an amine group. In Pseudo-schiff  
615 reactions, imine and aminal groups of chemical dyes react with aldehydes of the tissue,  
616 inducing a color change. Five classes of chemical dyes were defined according to the  
617 chemical reaction [87]. Non-fluorescent dye except combining with propidium iodide (mPS-  
618 PI).

619 **Point Spread Function (PSF):** mathematically described the image of point through an  
620 optical system and is the result of light diffraction and interference. PSF is represented by a  
621 3D interference pattern. The PSF size is defined by its intensity maxima :  $\frac{0,61\lambda}{NA}$  in lateral  
622 direction ( $x,y$  plan);  $\frac{2\lambda n}{(NA)^2}$  in axial direction ( $x,z$  plan) ( $NA$  : numerical aperture of the  
623 objective,  $\lambda$  : light wavelength,  $n$  : RI of the objective lens). According to Reyleigh criterion,  
624 these sizes give the distance that two points have to be separated by to be resolved. As PSF  
625 is larger in the axial direction than in the lateral direction (where it respects an Airy  
626 diffraction pattern), the lateral resolution of an optical system, is better than its axial  
627 resolution.

628 **Refractive Index (RI):** is a dimensionless value, often write  $n$ , indicating the deviation of light  
629 encountering a material.  $n$  is defined as the speed of light in vacuum ( $c$ ) divided by the speed  
630 of light in transparent material ( $v$ ),  $n = \frac{c}{v}$ .

631 **Stimulated Raman Scattering (SRS):** belonging, with Coherent anti-Stokes Raman Scattering  
632 (CARS) to Coherent Raman Scattering (CRS) microscopy. Here, a sample is simultaneously  
633 excited with two laser beams. When the difference of frequency between the two beams  
634 corresponds to the frequency of a chemical bond vibrational, there is an amplification of  
635 Raman signal (4 times) that can be detected [88]. It leads to label-free imaging of molecules  
636 and can also be combined with Raman tags (*e.g.* deuterated molecules) [89].

637

**Table 1. Comparison of optical clearing methods applied to plant tissues.**

Protocol name : Clearing Agent	Plant species	Organ	Refractive index of clearing solution	Protocol duration	Chemical treatments	Enzymatic treatment	Staining	Immuno staining	Microscope	Software	Reference	Reference on animal	Advantages/disadvantages	
<b>Organic solvents</b>														
Chloral hydrate, chloroform	<i>A. thaliana</i>	Leaves Seedlings	1.47-1.48	days	Fix. in ethanol acetic acid; perm. NaOH/SDS	Amylase	mPS-PI	No	CLSM, multiphoton	ImageJ, MedINRIA ImageViewer	[12]	[22]	microscope comparison reported/ reagent toxicity, staining step could eliminate protein and nucleic acid	
PF, PP11	<i>A. thaliana</i>	Leaves	1.31	minutes	No	No	SEYFP	No	CLSM + airyscan, two-photons, second harmonic generation, SRS	Zeiss510	[24]	unknown	fast protocol, suitable with a lot of microscopy type/ never re-used in literature	
Visikol : confidential	Various	Leaves, Roots	1.44	minutes/ hours	No	No	GUS staining, periodic Acid, Schiff 's reagent, PI, CyclinB1;1-GUS, FM4-64, GFP, Brefeldin A	No	Fluorescent microscope, CLSM, CLSM+airyscan	SP7, Zeiss Zen, ImageJ	[26-28]	[91]	easy to use/ no referenced for volumetric imaging	
BABB : benzyl alcohol/benzyl benzoate (or Murray's clear)	<i>A. thaliana</i> , various with or without insect	seedlings, leaves, flowers, fruits	1.56	days/ week (months for insect)	Fix. acetone, deh. methanol	No	GUS staining, autofluorescence	No	M-OPT	Matlab, NRecon, VolViewer, Driшти	[30]	[6,92]	very large samples (cm order)/ no FP imaging reported	
	<i>A. thaliana</i> , <i>Medicago truncatula</i>	galls, syncytia, nodules, cysts, egg masses		hours/ days	Fix. 3% glutaraldehyde; deh. ethanol	No	(glutaraldehyde autofluorescence)	CLSM	LCS	[31]	efficient for clearing dense tissue/ no FP imaging reported			
FluoclearBABB : BABB	Mouse	brain	1.56	hours/ days	Fix. 4% PFA; deh. propanol or tert-butanol; basic pH	No	eGFP	No	LSFM, CLSM	Fiji	unknown	[33]	FP imaging with BABB/not yet applied on plant tissue	
Methyl salicylate	<i>Zea mays</i>	ovaries, delopping seeds	1.538	days	Fix. FAA (various concentrations); deh. ethanol	No	Feulgen staining (acriflavine)	No	CLSM	unknown	[35]	[6,34]	fast protocol/ unsuitable with endogenous FP	
	Crassinucellate	flower buds, mature flowers, immature fruits		one day	Fix. FA; perm. acetone; deh. isopropanol	No	rhodamine-phalloidin, Hoescht	No	CLSM	LASX	[36]			
	Various crop species	Root		days	Fix. 4% PFA, 5% DMSO; deh. ethanol	RNase	PI, GFP	No	stereomicroscope, CLSM	LASX	[37]			
<b>Simple immersion in aqueous solutions</b>														
SeeDB : fructose, $\alpha$ -thioglycerol	<i>Acer truncatum</i> , <i>Platanus occidentalis</i> , <i>Robinia pseudoacacia</i> , <i>A. thaliana</i>	Leaves	1.52	days	Fix. FAA (38% FA); deh. in ethanol	No	PI, Calcofluor white	No	CLSM	Bitplane's, Imaris	[39]	[38]	no clearing effect reported	
NaOH			unknown										[93]	tiny effect, no transparency
NaClO			unknown										unknown	efficient for depigmentation/ no information concerning tissue content impact
2,2'-thiodiethanol (TDE)	<i>A. thaliana</i>	Various	1.42 - 1.52	hours	Fix. 4% PFA	No	GFP-Tdtomato	No	CLSM, two-photons	Fiji	[45]	[41,43]	refractive index linearly proportional to TDE/ inefficient to remove pigments	
TOMEI : TDE	<i>A. thaliana</i> , <i>Oryza sativa</i>	Various	1.515	hours	Fix. (TOMEI-I) acetic acid : ethanol (TOMEI-II) 4% PFA	No	DAPI, SYBR green I, GFP	No	CLSM+spinning disk, two-photons	ImageJ, FluorRender, Fluoview, MorphoGraphX, MetaMorph	[44]			

<b>ITOMEI:</b> iohexol, caprylyl sulfobetaine	<i>A. thaliana</i> , <i>Oryza sativa</i> , <i>Marchantia</i> <i>polymorpha</i>	Seedlings, leaves, shoot apical meristem, gemma, gemma cup	1.518	one day	Fix. 1% FA	No	various endogenous FP, SR2200, Calcofluor White M2R, Evans blue	No	fluorescence stereomicroscope, CLSM, two- photons,	ImageJ, LASX, Imaris	[46]	[47]	efficient to remove pigments/ could impact lipid and protein content
<b>Protein hyperhydration</b>													
<b>ScaleP</b> , <b>Warner's</b> <b>protocol</b> : urea, Triton X100	Various	Various	unknown	days/ weeks	Fix. 4% PFA ; Glutaraldehyde	cellulase, pectolyase (for immulabelling only)	Alexa fluor 594, Calcofluor white, SYTO 13, Citrine- GFP	Yes : anti-b- 1,3- glucan (nodules )	Multiphoton, CLSM	Amira 3D, Huygen's	[49]	[48]	based on a systematic chemical compound screening, suitable with a wide range of plant species and organ/ time to clear
<b>ClearSee</b> : urea, sodium deoxycholate , xylitol	<i>A. thaliana</i> , <i>Physcomitrella</i> <i>patens</i>	Seedlings, leaves, pistils, gametoph ores	1.41	days/ weeks	Fix. 4% PFA	No	Various endogenous fluorescent proteins	No	CLSM, Two photons	NIS- Elements AR 4.10 (Nikon) ZEN 2010	[15]		
<b>ePro- ClearSee</b> : idem ClearSee	Various	Leaves, roots			Fix. 3% PFA; delipidation 0,3% Triton X100	cellulase, pectolyase	DAPI	Yes : various antibodi es	CLSM + airyscan	Zen 2 '	[56]		
<b>ClearSeeAlpha</b> : ClearSee + sodium sulphite	Various	Leaves	unknown		Fix. 4% PFA	No	mClover	No	Multiphoton, CLSM	NISElemen ts	[57]		
<b>CTR</b> : ClearSeeAlpha	<i>Vitis vinifera</i>	Roots		weeks	Fix. 4% PFA, 5% DMSO	cellulase, pectolyase	PI	No	CLSM	LASX	[59]		
<b>Tissue transformation</b>													
<b>PEA-CLARITY</b> : SDS, Boric acid, NaOH	<i>A. thaliana</i> , <i>Nicotiana</i> <i>tabacum</i>	leaves	unknown	weeks/mo nths	Fix. 4% PFA; hydrogel embedding	cellulase, xylanase, arabinofura nosidase, pectatolyas e, $\alpha$ - amylase	PI, Calcofluor white	Yes : antiRubi sco	CLSM	LAS-AF	[61,62 ]	[60]	tissue permeabilization and stability/ time to clear

Fix. : fixation, deh. : dehydration, perm. : permeabilization, PFD : PerFluoroDecalin, CLSM : confocal laser scanning microscope

640 **Figures**

641 **Figure I, Box1. Fundamental principles of optical clearing process**

642

643 **Figure 1. Key values of refractive indices.** Refractive indices key values. Plant cell  
644 components (left) cover a wide range of refractive indices (RI, dimensionless), rendering  
645 plant tissue very scattering and challenging to image in photonic microscopy. In order to  
646 homogenize RI into plant tissue, diverse clearing agents have been used (right). (TDE : 2,2'-  
647 thiodiethanol; SeeDB : See Deep Brain; BABB : Benzyl Benzoate/Benzyl Alcohol).

648

649 **Figure 2. Taxonomy of clearing protocols for plant tissues.** Clearing protocol taxonomy for  
650 plant tissues. Adapted from the last update of clearing protocol taxonomy of animal  
651 research literature [5], implemented by the fluorescent staining employed. (PFD:  
652 PerFluoroDecalin; PP11: perfluoroperhydrophenanthrene; TDE: 2,2'-thiodiethanol; TOMEI:  
653 Transparent plant Organ MMethod for Imaging; iTOMEI: improved TOMEI; ePro-ClearSee:  
654 enzymes 2-Propanol ClearSee; CTR: Clearing Tannic Roots; PEA-CLARITY : Plant-Enzyme-  
655 Assisted-CLARITY). Description of each protocol in the main text.

656

657 **Box 1. Clearing process**

658 Plant tissues present a wide diversity of cell types (*e.g.* parenchyma cells, epidermis cells),  
659 organelles (*e.g.* chloroplasts, amyloplasts) and components (*e.g.* waxes, pigments). Each of  
660 these has its own optical properties and can impede the path of light through the tissue  
661 layers. Two major optical phenomena are involved: absorption and scattering.

662 Light absorption is a loss of photons: their energy is absorbed by molecules. Pigments absorb  
663 in the visible spectrum and are more represented in plant kingdom than in any other  
664 kingdom. A key process in the clearing of plant tissue consists in reducing absorption by  
665 eliminating pigments (Figure I).

666 Light scattering is the deviation of photons from their straight trajectory when they  
667 encounter a medium with a different **Refractive Index (RI)**. It is a very important parameter  
668 in optical fluorescence microscopy, and it is described by the law of refraction. Thus, if a  
669 photon is too deviated, it cannot be detected and the signal is lost. In plant tissues, RI range

670 from 1.00 (air cavities) to 1.61 (lignin [17]). Consequently, the thicker the sample, the higher  
671 the scattering events. Hence, in-depth observation is limited to 50  $\mu\text{m}$  in leaf tissue [15]. The  
672 major process of the CP is the homogenization of RI throughout the entire tissue (Figure I).

673 Because the optical properties of a tissue are dependent on its specific composition, CPs  
674 have to be optimized not only according to species (*e.g.* moss  $\neq$  tree), but also according to  
675 tissues (*e.g.* leaf  $\neq$  root), and to microscopy techniques (see section 2 and Box 2).

676

### 677 **Box 2. A focus on light sheet fluorescence microscopy**

678 The development of **Light Sheet Fluorescence Microscopy (LSFM)** was a breakthrough  
679 innovation in optical sectioning [94]. In LSFM, the specimen is illuminated by a sheet of light  
680 perpendicular to the detection system, and the centre of the light sheet matches the focal  
681 plane. The main advantage is that fluorophores outside this plane are not illuminated, so  
682 that noise and phototoxicity are lower. But, the system is highly sensitive to scattering and  
683 absorption because photons emitted in the focal plane must travel through the rest of the  
684 tissue before reaching the objective. Moreover, a specific mounting system is required  
685 regarding the angle of illumination, and mostly involves fixing a piece or an entire specimen  
686 onto a support.

687 This planar illumination combined with a fluorescent signal were first developed under the  
688 name Orthogonal-Plane Fluorescence Optical Sectioning (OPFOS [95]). Faced with the  
689 opacity of bones, the first guinea pig cochlea observed under a light sheet microscope was  
690 cleared using Spalteholz clearing agent. This first LSFM was developed with a CP, promising  
691 joined developments between microscopy techniques and sample preparation. A device  
692 combining perpendicular illumination plant and point scanning confocal microscopy was  
693 developed and gave a better axial resolution than ordinary confocal microscopy [96]. In  
694 2004, Huisken and colleagues [97] optimized their instrument by using wide-field detection  
695 instead of confocal detection: Selective Plane Illumination Microscopy (SPIM) was born, and  
696 modern LSFM in its wake. Meanwhile, the popularity of LSFM increase and developments of  
697 the technique stemmed from homebuilders scientists and instrumentals companies to the  
698 point that in 2014, LSFM was recognized as “method of the year” by the journal *Nature*.

699 LSFM implementations concerned illumination and detection optics (*e.g.* two sides  
700 illuminations, two detection paths, upright or inverted, four paths used at detection and  
701 illumination, multidirectional illumination). Moreover, standard Gaussian beam light sheet  
702 has been modified to enhanced LFSM performances (*e.g.* digitally-scanned laser light-sheet,  
703 light coherence controlled; coupled with two-photons or multiphoton illumination; replaced  
704 by self-reconstructing beams, lattice light sheet, infrared light sheet) [98]. These  
705 enhancements lead to the homogenization of illumination; corrections of the shadow effect  
706 and stripes artefacts; increasing imaging speed, resolution and depth penetration. In 2020,  
707 Masters [99] published an insightful book about super-resolution microscopy in which  
708 advances in LSFM were well described and discussed. Moreover, numerical post-processing  
709 treatments exist to improve image quality [98]. Among all LSFM set-up available nowadays,  
710 it is really important to choose one that suits the biological cleared sample and the  
711 experimental interest [100].

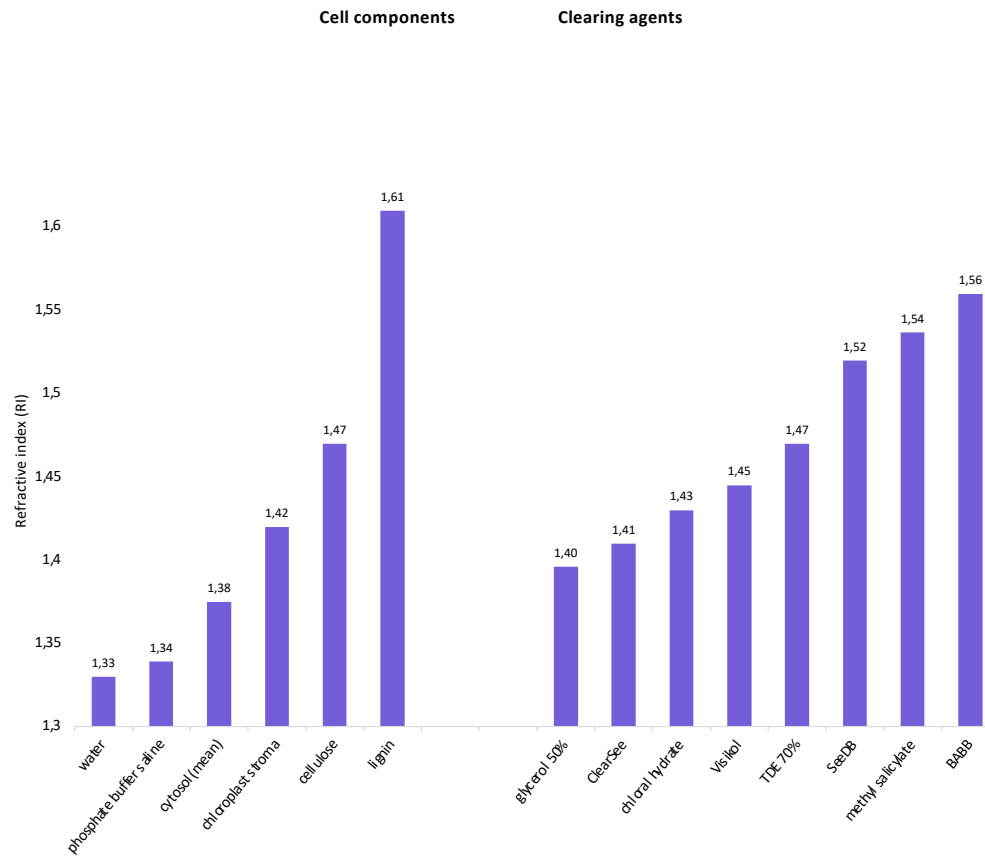
712 Concerning plant research, a review by Ovečka and colleagues [101] presents and discusses  
713 plant development imaging by LSFM. Most of publications on plant tissue imaged by LSFM  
714 concern *in vivo* acquisition. Even though some publications are now dedicated to LSFM on  
715 cleared samples [102], none has yet addressed cleared plant tissues.

716

717



Figure 1



# Clearing protocols for plant tissues

Organic solvents

PFD, PP11

Visikol

Methyl salicylate

Immersion in aqueous solutions

NaClO

TDE

TOMEI

iTOMEI

Hyperhydrating solutions

Warner's protocol

ClearSee

ePro-ClearSee

ClearSeeAlpha

CTR

Tissue transformation

PEA-CLARITY

