Chapter 1

Monitoring Photosynthesis by *In Vivo* Chlorophyll Fluorescence: Application to High-Throughput Plant Phenotyping

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Abstract

In spite of the decrease in the rate of population growth, world population is expected to rise from the current figure (slightly above to 7.2 billion) to reach 9.6 billion in 2050. There is therefore a pressing need to increase food production. Since most of the best arable lands are already under production, expanding the agricultural areas would have negative impacts on important natural areas. Thereby, increasing the productivity of the current agricultural areas is the chief objective of agronomical planners, and planting more productive and better adapted plant varieties is crucial to achieve it. In fact, plant breeding is at the forefront of concern of both agronomists and plant biologists. Plant breeding is a millenary activity that deeply changed our world. However, the use of molecular biology techniques jointly with informatics capabilities—giving rise to the omics techniques—deeply accelerated plant breeding, providing new and better plant varieties at an increased pace. The advances in genomics, though, far by-passed the advances in phenomics, and so there is a rising consensus among plant breeders that plant phenotyping is a bottleneck to advancing plant breeding. Therefore, a range of international initiatives in high-throughput plant phenotyping (HTPP) are at course, and new automated equipment is being developed. Phenotyping plants, however, is not a simple matter. To begin with, it has to be decided which parameters to measure in order to extrapolate to the desired goals, plant resistance and plant productivity. For this, as well as for plant breeding, an in-depth knowledge of plant physiology is required. Photosynthesis has been considered as a good indicator of overall plant performance. It is the only energy input in plants and thereby impacts all aspects of plant metabolism and physiology. The cumulative rate of photosynthesis over the growing season is the primary determinant of crop biomass. It largely determines the redox state of plant cells, and therefore, it is at the core of regulatory networks. Therefore, assessing photosynthesis and the photosynthetic apparatus plays a core role on plant phenotyping. Nevertheless, high-throughput phenotyping demands very rapid measurements, and consequently the most common method of photosynthesis measurement—the infra-red gas analysis—is not well suited for this purpose. On the contrary, the techniques based on *in vivo* chlorophyll (Chl) a fluorescence measurements are perfectly fit. In this chapter, an historical perspective on the development of *in vivo* Chl a measurement is briefly addressed. Then, the state of the art of the fluorescence-based techniques of photosynthesis assessment is presented, and their potential use in HTPP is evaluated. Finally, the current use of these techniques in the main systems of phenotyping is surveyed.

Keywords: photosynthesis, photochemistry, chlorophyll fluorescence, optical techniques, plant phenomics, high-throughput plant phenotyping

1. Introduction: Food security, plant breeding, and high-throughput plant phenotyping

Albeit the rate on population rise is slowing down, world population is still increasing; from the current figure slightly above 7.2 billion, it is expected to reach 9.6 billion by 2050 [1]. Therefore, there is a pressing need to increase the global food production ([2] and references therein). Mostly, the population increase is expected to occur in developing countries ([3] and references therein), where the current productivity of farms is far below the one in the developed world. For instance, in India, where the population is expected to surpass China's, the current productivity of wheat farming is only one third than that of France, while rice productivity accounts for less than half of China's [4]. Increasing food production and ensuring availability of safe and nutritious food at affordable prices to the population of developing countries are, therefore, a pressing urgency. As most of the best arable lands are already under production, expanding the agricultural areas would have negative impacts on important natural areas. Thereby, increasing the productivity of the current agricultural areas is the chief objective [5], which requires more productive and better adapted plant varieties [6]. There is a long tradition of conventional plant breeding that deeply changed our world [7]. Moreover, being a cost-effective tool for increasing nutritional value of forage and crops, plant breeding still can contribute to global food security [4]. Therefore, it is at the forefront of concern of both agronomists and plant biologists. The use of molecular biology techniques jointly with informatics capabilities—giving rise to the omics techniques—deeply accelerated plant breeding, providing better plant varieties at an increased pace [8]. In fact, during the past 20 years, molecular profiling and classical sequencing technologies enabled significant advances toward the large-scale characterization of plant genomes [9], yielding valuable tools for plant breeding such as marker-assisted selection [10]. However, integrating approaches across all scales, from molecular to field applications, are necessary to develop sustainable plant production with higher yield. Regrettably, the advances in genomics far by-passed the advances in phenomics, and therefore, there is a rising consensus among plant biologists that plant phenotyping is a bottleneck to advancing both fundamental research and plant breeding [11]. Therefore, a range of international initiatives in high-throughput plant phenotyping (HTPP) are at course, and new automated equipment is being developed [12]. Plant phenotyping is "the application of a set of methodologies and protocols used to measure plant growth, architecture, and composi-

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tion with a certain accuracy and precision at different scales of organization, from organs to canopies" [11]. Phenotyping, being a paradigm of the interdisciplinary character of modern plant physiology [13], is not a simple matter. To begin with, it has to be decided which parameters to measure to extrapolate to the desired plant traits (resistance and productivity). That is, researchers need sound and robust knowledge about the traits that are indicative of the intended performance. Here, mechanistic understanding of plant physiology plays a role in identifying useful parameters and proxies to measure [11]. In any case, it seems plausible that effective HTPP platforms will involve the measurement of multiple parameters. Two classes of parameters are of major importance: structural parameters and photosynthetic parameters. The structure of both the shoots [14] and the roots has been primarily phenotyped using a broad range of cameras sensitive in the visible spectral range. Conversely, photosynthetic activity has been phenotyped mainly by using *in vivo* chlorophyll (Chl) fluorescence techniques.

2. Photosynthesis: a proxy of plant performance

An in-depth knowledge of plant physiology is required for successful plant breeding [15], as most of plant stresses are under the control of complex traits [16]. In particular, as accumulating data did not fit into conventional theories, it becomes clear that metabolic engineering is in need of a better understanding of metabolic regulation and plasticity [8]. Photosynthesis has been considered as a good indicator of overall plant performance. It is the only significant energy input in plants and thereby impacts all aspects of plant metabolism and physiology. The cumulative rate of photosynthesis over the growing season is the primary determinant of crop biomass [17]. Photosynthesis largely determines the redox state of plant cells and therefore is at the core of regulatory networks [18]. Therefore, assessing photosynthesis and the photosynthetic apparatus plays a core role on plant phenotyping. Nevertheless, high-throughput phenotyping demands very rapid measurements, and consequently, the most common methods of photosynthesis measurement—the infra-red gas analysis and the polarographic measurement of oxygen evolution—are not well suited for this purpose. On the contrary, optical methods present considerable advantages for *in vivo* and *in vitro* assessment of the physiological condition of live tissues, as compared to chemical and physicochemical methods, because they are much faster, non-invasive, and non-destructive (e.g. [19, 20]). Among these, *in vivo* Chl fluorescence measurements are best suited for this purpose. Albeit the use of optical methods in plant phenotyping has been recently reviewed [21], to our knowledge, a review specifically addressing the use of Chl fluorescence techniques in HTPP is missing in the literature. In the following sections, an historical perspective on the development of *in vivo* Chl fluorescence measurement, from the seminal work of Kautsky and Hirsch [22] to the ground-breaking invention of pulse amplitude modulation (PAM) [23], is briefly addressed. Then, the current state of the art of the Chl fluorescence measurement techniques is presented, and their potential use in HTPP is evaluated.

3. Chlorophyll fluorescence

The emission of photons from excited molecules was named fluorescence by the Irish physicist George Gabriel Stokes [24], after fluorspar or fluoride, the mineral from calcium fluorite where he studied the phenomenon. When a molecule of Chl a from the antenna complex of a photosynthetic organism is hit by a photon, it absorbs its energy and an electron is raised to a higher energy level S1 (singlet 1, corresponding to the absorption of one red photon) or S3 (singlet 3, corresponding to the absorption of one blue photon; **Figure 1**).

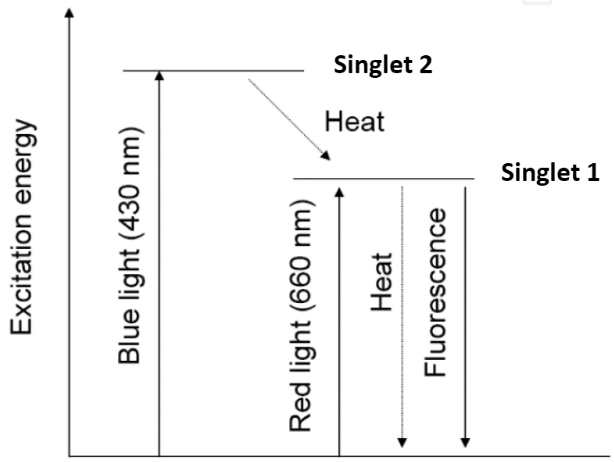

Figure 1. Simplified diagram of the energy levels of the singlet excitation states of chlorophyll a molecules. *Source*: Adapted from Ref. [25].

The excited molecule is very unstable, and its excess energy is promptly released. There are three different competing ways of de-excitation: (1) heat dissipation; (2) photochemical utilization of energy; and (3) fluorescence emission. The relative contribution of each process is dependent on the physiological status of the photosynthetic systems [25]. In the last few decades, the measurement of Chl fluorescence has become a universal technique in the study of virtually all types of photosynthetic entities, including fruits [26–28], corals [29], seagrasses [30], macroalgae [31], microphytobenthos [32–34], and many types of higher plants, such as tobacco [35], maize [36], and tomato [37]. The use of Chl fluorescence has been recently proposed for detecting early responses to abiotic and biotic stresses, before a decline in growth can be observed [38–40]. Likewise, there are numerous applications of Chl fluorescence in the horticultural sectors (reviewed in [41]). With the advent of different instrumental techniques, Chl fluorometry developed into various types, with different timescales of signal capturing [42]. It is useful, however, to divide the currently available techniques for *in vivo* Chl fluorescence measurements into passive and active [43]. While passive techniques measure fluorescence emission under actinic light, active techniques stimulate fluorescence emission using dedicated light sources.

3.1. Passive fluorescence

Albeit it has been known for a long time that Chl, like many other molecules, emits fluorescence after excitation, it was not until 1931 that Kautsky and Hirsch observed that the *in vivo* emission of fluorescence during a dark–light transition showed a typical variation [22], usually known as the Kautsky induction curve or simply Kautsky effect. Even though these authors have speculated on a possible relation between the observed fluorescence emission and carbon fixation, the molecular basis of photosynthesis was, at the time, poorly understood,[1] and therefore, their observations did not significantly impact photosynthesis research. As the understanding of the molecular mechanisms progressed, however, prototypes of continuous fluorescence recording fluorometers were built and used in photosynthesis research [45]. These simple devices had limited use in stress physiology, but Lichtenthaler and Rinderle [46] developed the Vitality Index (Rfd = $F_v'/(F_m–F_v')$) and successfully used it to detect low temperature stress in higher plants. However, continuous fluorescence recording fluorometers acquired increase potential in photosynthesis research only when equipped with high-time resolution capacities. This allowed to explore the kinetics of the fast phase of fluorescence signal rise in a dark–light transition. Although the exploration of these signals begun earlier with experimental prototypes, it was the commercial availability of the Plant Efficiency Analyzer by the UK-based manufacturer Hansatech that made this technique widely available to plant physiologists and plant breeders. In Switzerland, Reto Strasser provided the theoretical basis for the interpretation of these signals [47]. His group at the University of Genève have developed the JIP test (termed after the main inflections in the fast fluorescence rise, called J, I, and P) to analyze the photosystem II (PS II) behavior [48] (**Figure 2**).

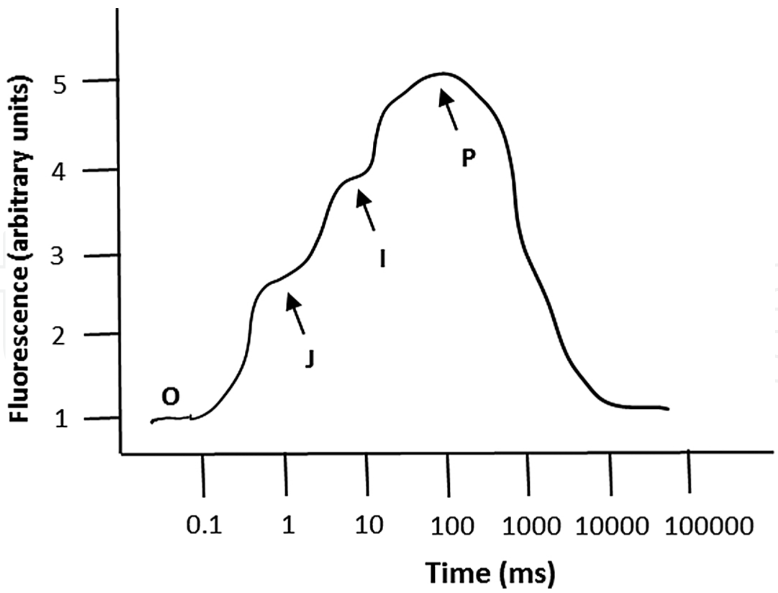

Figure 2. Chlorophyll fluorescence induction curve (OJIP transient).

[1] Please note that the very concept of photosynthesis as being a redox process was only to be demonstrated 6 years later [44].

Based on the Chl a fast fluorescence transient measurement [49], numerous indexes and parameters quantifying the energy flow related to the different phases in the PS II photochemical reactions can be calculated. The JIP transient rise reflects the successive but overlapping reduction of the electron acceptor pool of PS II [50] and can be used to obtain information on the redox state of the photosynthetic electron transport chain, on the stoichiometry of its components and on the relative PS II antenna size [51]. This transient has been found to be very sensitive to stress caused by changes in different biotic and abiotic conditions, presenting alterations even before visible symptoms could be detected on the plants [52–57]. Albeit some information may be obtained from the application of the JIP test to pre-illuminated leaves, the most used protocols require a dark-adaptation period [58]. This may be achieved for many leaves simultaneously by using the leaf clips provided by the manufacturer Hansatech. Individual measurements are rapid as usually a 1-second light (saturating) pulse is applied, and the kinetics of fluorescence rise is immediately recorded. Therefore, the main hindrance to the use of the JIP test on high-throughput automated plant phenotyping is the need to previously dark-adapt the samples (**Table 1**). Albeit this technique has been proved useful in manual low-throughput plant phenotyping [57, 59], none of the commercial phenotyping platforms makes use of it. It is possible to envisage, however, a system where whole plants would be pre-adapted for dark conditions, and non-contact measurements of the fluorescence induction curve would be made. In fact, imaging of the JIP parameters is already possible and has been used to screen wild barley genotypes under heat stress [60]. Passive fluorescence spectra of leaves may also be obtained and provide information on the status of the photosynthetic apparatus [61]. However, fluorescence emission spectra have been studied mainly with active fluorescence techniques, mostly with laser-induced fluorescence (LIF) [62] which is discussed in the next section.

3.2. Active fluorescence

3.2.1. ***Laser-induced fluorescence***

Since LIF measurements can be carried out remotely, allowing, for example, to inspect difficult-to-access canopies, this technique is particularly interesting for HTPP in the field. In fact, large crop areas can be efficiently surveyed by scanning an instrument placed at a high viewpoint or by mounting it in an airplane or a drone [63]. LIF was applied for estimating the overall metabolic activity of plants during a defined period of time [64, 65]; differentiating plant species [66–68]; assessing potassium deficiency [69]; estimating the maturity of lettuce [70]; detecting mildew and rust fungal [71] and bacterial [72] infections; and studying the influence of water stress [73, 74], ambient light [68], UV radiation [75], atmospheric [76], and soil pollutants [76, 77] and excess of ammonium nitrate [78] and nickel [79] on plant physiology. In addition, LIF has been used to assess the productive biomass of benthic diatoms [62, 80] and to differentiate between groups of macroalgae [81]. LIF spectra of plant leaves present a local Chl emission maximum in the red region of the spectrum at 685 nm (F685) and an absolute maximum at the far-red region, circa 740 nm (F740) [65, 75]. The relative intensity, shape, and wavelength of these peaks are dependent on the physiological status of the photosynthetic apparatus. Changes in the Fr/Ffr ratio were suggested to be well correlated to Chl a concentration [69, 82, 83], which is altered, in most plant species by stress [84]. In addition to changes in the Chl concentration, other factors, related to membrane lipid composition and protein environment [85] as well as leaf ultrastructure and the accumulation of specific light absorbing metabolites, such as anthocyanins, are likely to affect Fr/Ffr ratios. It is known that water stress significantly impacts leaf lipid composition of thylakoid membranes, causing a decrease in the contents of polar lipids, namely chloroplast-specific glycolipids as well as changes in their fatty acid composition, likely to affect membrane fluidity [86]. In forest species subjected to severe drought, Lavrov and coworkers [73] showed that the red/far-red emission fluorescence ratio (Fr/Ffr) is very well correlated with the maximum potential photochemical efficiency of PS II estimated with a Plant Efficiency Analyzer (Hansatech, U.K.). Also, simultaneous measurement of Chl fluorescence in maize (*Zea mays*), sugar beet (*Beta vulgaris*), and kalanchoë (*Kalanchoë* sp.) by LIF and PAM indicated that the steady state of fluorescence is useful for water stress detection [87]. Albeit LIF has been extensively used in applied research, its possibilities have not been thoroughly explored in fundamental research. In fact, even though *Arabidopsis thaliana* had become the main model organism in plant biology, applications of LIF to this species are almost absent, the exceptions being a study on npq mutants, altered in the expression of the PS II PsbS protein [88] and, more recently, a study on the water stress effect on photosynthesis [74]. Advanced variations of this technique as is the case of laser-induced fluorescence transients (LIFT) allow the calculation of quantum efficiency [89], leading values in line with the ones obtained by the more established technique of PAM fluorometry (see below).

3.2.2. ***Conventional pulse amplitude modulated fluorescence***

Active fluorescence protocols exploiting PAM [23] can measure the potential and effective quantum efficiency of photosystem II, the electron transport rate, and the extent of non-photochemical quenching. Based on the concepts used in the light-doubling technique [90], PAM fluorometry enables to distinguish between the photochemical (qP) and non-photochemical (i.e., dissipative; qN and NPQ) use of light energy. Notably, the quantum efficiency of photosystem II can be measured much more easily than the other parameters [91]. Kitajima and Butler [92] showed that the maximum potential quantum yield of PS II is characterized by the dimensionless parameter F_v/F_m (the ratio of variable and maximum fluorescence measured after saturating light pulses). A very constant value of 0.832 ± 0.004 was found for healthy leaves of a very wide variety of species [93] while stress due to disease or environmental conditions is indicated by lower values. The basal fluorescence (F_o) is dependent on the tissues' Chl concentration [33], which, in turn, depends on the physiological condition of the photosynthetic system. However, severe stress may change the basal fluorescence yield, affecting the relation between F_o and Chl concentration. In fact, a significant increase of F_o due to heat stress, independent of the Chl concentration was reported by Havaux and Strasser [94]. The heat-induced increase of the basal fluorescence intensity probably reflects a disturbance on the organization of thylakoid membranes [95]. The F_v/F_m parameter appears to be relatively insensitive to severe water limitation but could be used to differentiate between responses during cold. On the basis of the calculation of the fluorescence index $\Delta F/F_m'$ (where ΔF is the difference between the maximal fluorescence [F_m'] and the steady-state fluorescence [F] of light-adapted samples), which measures the effective quantum yield of PS II [96], PAM fluorometry allows the construction of rapid light curves (RLCs) relating the rate of photosynthetic electron transport and incident photon irradiance [97, 98]. PAM was successfully applied to a wide range of plants, such as the olive tree, rosemary, and lavender [99]; *Paspalum dilatatum* [100], *Phillyrea angustifolia* [101], and other Mediterranean shrubs [102]; the tropical grass *Setaria sphacelata* [84] and several C4 turfgrasses [103]; maize [36, 104]; and *Arabidopsis thaliana* [105], among others.

3.2.3. *Imaging pulse amplitude modulated fluorescence*

The development of Chl fluorescence imaging systems by numerous research groups [106–108] together with the emergence of commercially available models by PSI (Brno, Czech Republic), Walz Systems (Effeltrich, Germany), and Technologica Ltd. (Colchester, UK) has greatly increased the versatility of Chl fluorometry techniques (reviewed in [109]). Systems that image at the microscopic level allow to measure PS II photochemical efficiencies from chloroplasts within intact leaves and from individual cells within mixed populations [107, 110]. On the other hand, lower resolution imaging systems allow the mapping of fluorescence parameters over large areas, making it a unique technique to study the spatial heterogeneity of the photosynthetic activity across an autotrophic surface [111–113]. Conventional and imaging techniques use different technologies, namely in the detection processes of the fluorescence signal: a photodiode or phototube in conventional PAM fluorometry and a charge-coupled device (CCD) camera in imaging-PAM fluorometry. Consequently, caution is needed when comparing results from conventional and imaging fluorescence techniques [114]. Nevertheless, imaging-PAM fluorometry has proven to be a powerful technique and new technological developments, as the use of semi-automated systems equipped with fluorescence cameras continuously assessing the photochemical activity of leaves [115] are in course.

4. High-throughput plant phenotyping platforms and chlorophyll fluorescence techniques

The advancement of plant phenotyping is a key factor for the success of modern plant breeding and basic plant research. Since the recognition of the phenotyping bottleneck to plant breeding [12], a global effort to provide HTPP platforms was set on. Currently, most platforms are user-built, but some commercial platforms are already available, as is the case of the German platform from the manufacturer LemnaTec and the Czech platform from the manufacturer Photon System Instruments (PSI). The Canadian-based firm Qubit Systems offers a modified version of the PSI platform. To provide high-throughput phenotyping capabilities to plant breeders, numerous user-built phenotyping facilities are organized in networks, the most prominent being the European Plant Phenotyping Network, which offers access to 23 different plant phenotyping facilities to the user community [116]. Some countries organized national networks, as is the case of the German Plant Phenotyping Network [117] and the UK Plant Phenomics Network [118]. The Jülich Plant Phenotyping Centre [119] is a leading EPPN member. Jülich's platform makes use of LIFT to perform middle-range remote sensing of crops [119]. The commercial phenotyping platforms do not use LIF technologies. A simplified JIP test (restricted to the calculation of F_v/F_m) is used in the commercial platform from LemnaTech; a LemnaTech module with this feature is incorporated in the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) HTPP Platform [120]. PlantScreen, the commercial platform from PSI, uses conventional PAM fluorometry to perform quenching analysis. This platform has been used to phenotype cold-tolerant pea (*Pisum sativum*) plants [121]. Kjaer and Ottosen [122] used six independent PAM fluorometers in a HTPP experiment to assess daily growth of field-grown *Brassica napus*. In this case, however, the PAM system was exclusively used to show that the near infra-red laser beam of a 3D laser scanning, used for phenotyping, had not a deleterious effect over the photosynthetic metabolism of the plants. Bellasio and coworkers [123] have used an imaging-PAM system based on a FluorCam camera (PSI) inserted in a user-developed setting to phenotype common bean (*Phaseolus vulgaris*). The French platforms based at Montpellier [124] use an imaging-PAM system based on Walz's devices. An important advantage of Chl fluorescence imaging is that it can be used to screen a large number of small plants simultaneously [125]. A recent advance introduced by Serôdio and coworkers [126] allows the rapid generation of light curves from non-sequential, temporally independent fluorescence measurements. This technique has the potential to bring the valuable information provided by fluorescence RLCs into the realm of HTPP. David Kramer's group, at the Michigan State University, is currently developing a multi-instrument platform entitled Dynamic Environmental Photosynthetic Imaging (DEPI) [127], with the aim of reproducing in phytotrons the dynamics of field conditions, while continuously recording multiple parameters related with them photosynthetic performance.

Technique	**Potential**	**Limitations**	**Current use**	
			User-developed	**Commercial**
JIP test	Very fast measurements; well-established technique; successfully used in low-throughput plant phenotyping, including in field conditions	Need of a dark-adaptation period; signal interpretation not always straight forward; plant contact required	IPK (LemnaTech module)	LemnaTech (limited to F_v/F_m)
LIF	Middle-distance remote sensing; suitable for field phenotyping; LIFT allows the calculation of quantum efficiency	Fluorescence spectra less informative than variable fluorescence Very limited use in model plants	JPPC	Unreported
Conventional PAM	Very informative, physiological interpretation well established	Most protocols need a dark-adaptation period; measurements possible only at close range	Unreported	PlantScreen (Photon System Instruments) [121]

Technique	Potential	Limitations	Current use	
			User-developed	Commercial
Imaging PAM	Allows mapping of the photosynthetic heterogeneity over an autotrophic surface; facilitates replication.	Most protocols need a dark-adaptation period; measurements possible only at close range Expensive and sensitive equipment	JPPC [123]; M3P	PlantScreen (Photon System Instruments)

Note: JPPC: Jülich Plant Phenotyping Centre [119]; IPK: Leibniz Institute of Plant Genetics and Crop Plant Research [120]; M3P: INRA—Montpellier Plant Phenotyping Platforms [124].

Table 1. Applications of chlorophyll a fluorescence techniques in high-throughput plant phenotyping.

5. Prospective

Chl fluorescence techniques will continue to play a major role on HTPP. Among these, imaging-PAM techniques will play a pivotal role, although specific cases will require different technological solutions. Moreover, field HTPP, which is expected to be fostered in the forthcoming years, will require technologies not dependent on sample dark adaptations and able to operate at medium-range distance, where the family of techniques based on LIF may play a role. Finally, the development of low-cost HTPP platforms [128], required to improve plant breeding in developing countries, is expected to make use of the less expensive Chl measurement techniques, namely passive fluorescence. On the other hand, high-technology in-house HTPP platforms are expected to make simultaneously use of different Chl fluorescence techniques, integrated in a systems approach to plant phenomics.

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