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Photosynthesis

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Chapter 1

Tools for Measuring Photosynthesis at Different Scales

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Abstract

Measurements of in vivo photosynthesis are powerful tools that probe the largest fluxes of carbon and energy in an illuminated leaf, but often the specific techniques used are so varied and specialized that it is difficult for researchers outside the field to select and perform the most useful assays for their research questions. The goal of this chapter is to provide a broad overview of the current tools available for the study of photosynthesis, both in vivo and in vitro, so as to provide a foundation for selecting appropriate techniques, many of which are presented in detail in subsequent chapters. This chapter will also organize current methods into a comparative framework and provide examples of how they have been applied to research questions of broad agronomical, ecological, or biological importance. This chapter closes with an argument that the future of in vivo measurements of photosynthesis lies in the ability to use multiple methods simultaneously and discusses the benefits of this approach to currently open physiological questions. This chapter, combined with the relevant methods chapters, could serve as a laboratory course in methods in photosynthesis research or as part of a more comprehensive laboratory course in general plant physiology methods.

Key words Photosynthesis, CO_2 exchange, O_2 exchange, Chlorophyll fluorescence, On-line mass spectrometry

1 Principles of and Perspectives on Measuring In Vivo Photosynthetic Flux

The challenge of quantifying photosynthetic rates in vivo lies in the unique substrates and products of carbon assimilation. Photosynthesis involves a series of interconnected reactions that sequentially convert light energy into chemical energy and then use this energy to reduce carbon into usable sugars as shown non-stoichiometrically as

$$CO_2 + H_2O \overset{\text{Light}}{\rightarrow} O_2 + \text{Glucose} \quad (1)$$

Critical information concerning the mechanisms and biochemistry of photosynthesis can be determined in isolated or reconstituted enzymatic systems and can be probed in vivo to gain an

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integrated understanding of how photosynthesis is regulated and contributes to plant growth. Here, we focus on methods that measure an intact photosynthesizing system and how the information gained can contribute to broader research questions. Since photosynthetic reactions occur in the aqueous phase and glucose is rapidly converted to other forms, many measurement techniques examine gas exchange (assimilation of CO_2 or production of O_2) to measure photosynthetic activity in vivo. Additionally, given the unique properties of light energy capture and subsequent conversion to short-term chemical storage as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), there exists an array of light-based biophysical probes (i.e., chlorophyll fluorescence and leaf spectroscopy) that can also be employed to monitor specific components of the light reactions non-destructively and under natural conditions.

Today, a wide array of instruments able to measure CO_2 and O_2 exchange and other light-based biophysical probes of the light reactions in vivo are commercially available and can be further modified to interface with additional analytical platforms (Fig. 1), as is the case with on-line isotopic analysis. The variety of available and user-friendly instrumentation means that photosynthesis is easier to measure today than at any previous time, a benefit not only to researchers who focus on photosynthesis but also to those from other fields who want the insight that in vivo measurements of photosynthetic fluxes can provide.

One such application extending the relevance of in vivo measurements of photosynthesis to other fields is in understanding the response of crop production to climate change. Models of canopy crop production often incorporate biochemical sub-models that describe the response of leaf carbon fixation using in vivo biochemical parameters [1–4]. The biochemical parameters used to build leaf-level models of photosynthesis in response to changing climate are derived from in vivo measurements of carbon assimilation, and these parameters can vary greatly among species [5–8]. While these parameters are constant within some species like tomato [9], they are significantly different when measured among rice cultivars [10] and can even vary temporally throughout a growing season as seen in wheat flag leaves [11]. Given the variability of these photosynthetic parameters temporally and among species, it is apparent that in vivo measurements of photosynthetic flux under field conditions are needed to provide the parameters for higher-order climate models. Fortunately, current instrumentation provides the portability needed to make these measurements possible.

Instruments and experimental setups for measuring photosynthetic flux can be characterized broadly by what parts of Eq. 1 they measure (viz., CO_2 exchange, O_2 exchange, or light-based biophysical probes, Fig. 1). They can be further sub-divided according to what scale they measure, varying from chloroplast suspensions to

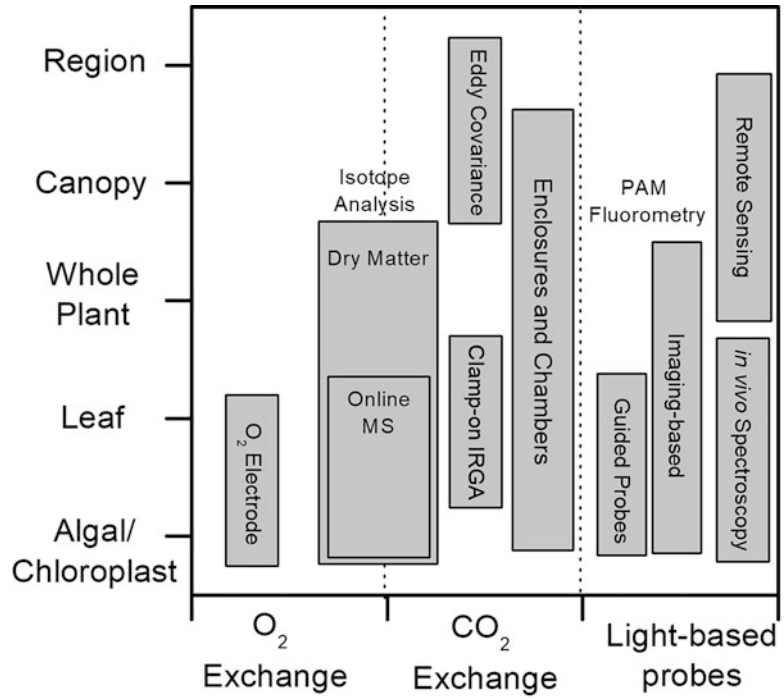

Fig. 1 Survey of tools used to measure photosynthetic fluxes discussed in this chapter. Shown are three different measurement principles (O_2 exchange, CO_2 exchange, and light-based biophysical probes) plotted against the different spatial scales at which measurements can be made for each technique, ranging from algal cultures to growing regions. Techniques plotted include O_2 electrode, isotope analysis including dry matter discrimination and online mass spectroscopy (MS), clamp-on Infra-Red Gas Analysis (IRGA), eddy covariance, enclosure and chamber-based analysis, Pulse Amplitude Modulated (PAM) Fluorometry (including guided approaches that use fiber optic cables or light sources in close proximity to the leaf surface and image-based approaches), in vivo spectroscopy for monitoring absorption shifts of wavelengths of interest, and remote sensing approaches using satellite or aerial imaging

regional land surfaces. Proper selection and use of these techniques depend on a correct understanding of the purposes, principles, advantages, and disadvantages behind each measurement.

The following section gives an overview of the major purposes, principles, advantages, and disadvantages of each of the methods discussed in later chapters. We also include additional techniques for completeness. Given the breadth of techniques described herein, our coverage will be necessarily brief with more comprehensive discussions available in the referenced work. We begin with gas exchange-based methods and then move to light-based probes of the photosynthetic light reactions.

2 Measurements of Gas Exchange

The observation that increased carbon fixation should drive greater plant production, and yield was the motivation for constructing the first system for measuring CO_2 exchange in an intact leaf [12]. Measurements of CO_2 were made possible with the development of commercially available Infra-Red Gas Analyzers (IRGA), which measure CO_2 concentrations via the absorbance of characteristic wavelengths in the infra-red spectrum. Measurements can be made in closed systems where the drawdown of CO_2 is measured in a closed cuvette or chamber as a function of time, or via open systems where CO_2 fluxes are resolved from the differences in gas concentrations in an air stream measured essentially before and after exposure to the plant material at a known flow rate. CO_2 fluxes can be determined at multiple scales to better understand the leaf-to-canopy interactions of the plant with the environment. Examples of commercial and custom-built systems used to measure CO_2 exchange at diverse scales reveal the wide variety of tools available to researchers today (Fig. 2). Note that the systems shown are only examples of commercially available instruments and neither intended to cover every manufacturer nor endorse a specific manufacturer. The reader is encouraged to consult the literature and contact a variety of manufacturers to determine which device(s) can provide the features needed for a specific research application.

Modern gas exchange systems based on IRGA technology combine CO_2 and H_2O measurements by making use of differences in the absorption spectra of CO_2 and H_2O. Estimating transpiration fluxes from differences in H_2O concentrations in the air is necessary to account for the dilution effect of transpired H_2O on the CO_2 concentration in the air and determine the intercellular CO_2 concentration. Gas exchange systems usually also measure and/or control other environmental conditions the leaf or plant is exposed to, such as temperature, light intensity, and air pressure. In combination, these parameters are used to derive the net CO_2 uptake or release of the measured plant.

2.1 Leaf CO_2 Exchange

Off-the-shelf gas exchange systems for measuring leaf gas exchange are readily available and increasingly user-friendly, which allows researchers to collect physiological measurements with the push of a few buttons. Leaf-level CO_2 exchange is routinely used to estimate instantaneous rates of net CO_2 assimilation (A_{net}) under ambient conditions (indicating the carbon uptake of the leaf at that moment) or under light- or CO_2-saturated conditions, allowing direct comparison of the CO_2 assimilation capacity between different leaves. Such measurements can be taken in a timespan on the order of minutes, allowing sampling of a large number of plants. A more detailed assessment of the biochemistry underlying the

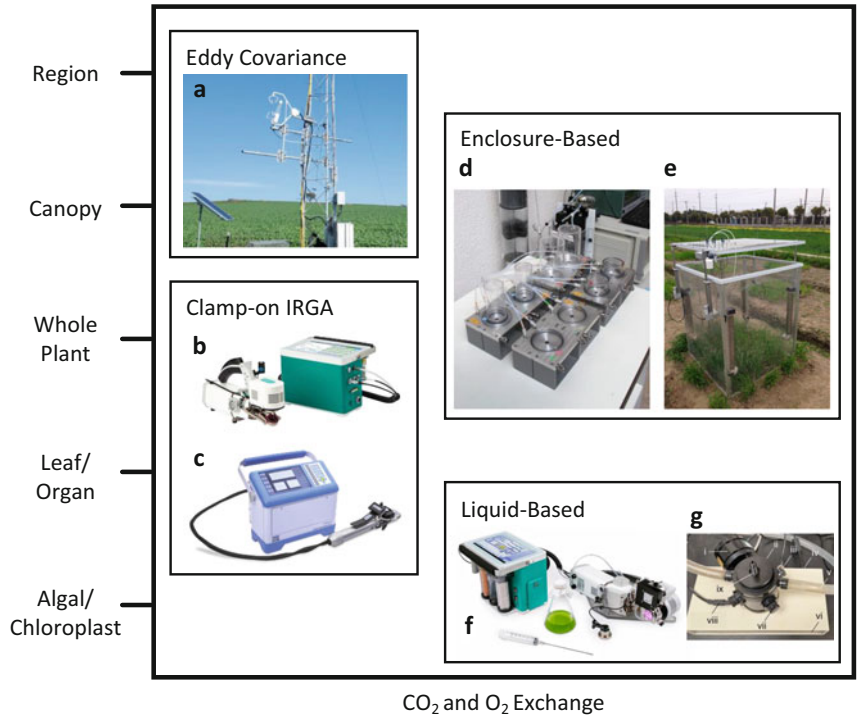

Fig. 2 Example tools used to measure CO_2 and O_2 exchange across diverse scales. At the canopy to the regional scale shown is a sample eddy covariance flux station (**a**). Commercially available portable CO_2 exchange systems for measuring leaf photosynthesis such as the LI-6800 from LI-COR Biosciences (**b**) and CIRAS-3 from PP Systems International, Inc. (**c**) can be used for leaf or whole plant measurements. For measurements of an enclosed canopy, we show the EGAS-2, a custom-built, multiplexed whole-plant gas exchange system (**d**), and the custom-built canopy enclosure of the CAPTS system (**e**). Measurements in liquid can be performed for either CO_2 exchange (e.g., with the LI-COR aquatic chamber; **f**) or O_2 exchange using a Clark-type oxygen electrode such as the Oxygraph+ System from Hansatech Instruments Ltd. (**g**). Instruments from the various manufacturers are shown only for illustrative purposes and do not imply any specific recommendation. The above images are reproduced with kind permission from their respective copyright holders: Caitlin Moore (**a**), LI-COR Biosciences (**b**, **f**), PP Systems International, Inc. (**c**), Gavin George (**d**), Qingfeng Song (**e**), and Steven Burgess (**g**)

photosynthetic processes of the studied leaf can be obtained by taking repeated measurements on the same leaf under varying CO_2 concentrations, light intensities, or temperatures. Measurements of A_{net} in response to the CO_2 concentration can be used in combination with a biochemical model of photosynthesis (such as the Farquhar, von Caemmerer, Berry (FvCB) model; [13]) to estimate, e.g., the maximum rate of Rubisco carboxylation (V_{cmax}) [14], the rate of electron transport (J), or the rate of phosphate limitation (TPU-limitation) [15]. Techniques to measure net gas exchange to resolve biochemical parameters such as V_{cmax}, J, and TPU-limitation are presented herein [16]. Similarly,

assessing how A_{net} changes with light intensity yields estimates of the maximum rate of photosynthetic electron transport (J_{max}; [17]) and of the light-saturated rate of photosynthesis [18]. In addition, measurements of transpiration can be used to quantify the diffusive barriers for CO_2 to enter the leaf via the stomata, termed stomatal conductance (g_s). These response measurements require more time to perform than those under a single condition, often more than an hour, but help elucidate whether differences between plants are due to the environment the plant is exposed to and the plant's transient response to it, or whether they are caused by some more long-term acclimation of its biochemistry or anatomy. In addition to these plant-specific parameters, gas exchange has been used to resolve Rubisco kinetics in vivo [19–21]. This approach provides values for some of the most important input parameters for the widely used FvCB model.

While some photosynthetic parameters are easily obtained with "standard" leaf-level gas exchange, more detailed information can be gained with more sophisticated setups. Stomata are often distributed unevenly between the two sides of a leaf, resulting in unequal contribution of the surfaces to CO_2 uptake [22, 23]. Gas exchange parameters can be estimated more accurately when accounting for cuticular conductance to water, which can be quantified when measuring photosynthesis on both leaf surfaces independently [24, 25]. A similar approach also allows the estimation of the CO_2 concentration at the surface of mesophyll cells, indicative of intercellular air space diffusion [26]. How to set up measurements of simultaneous and independent gas exchange on both sides of the leave is described herein [27].

Leaf-level gas exchange can also be combined with other techniques to gain further insight into plant physiology and metabolism. In combination with stable isotope measurements, it can be used to assess internal CO_2 diffusion [28] or to quantify gross fluxes of CO_2 into and out of the leaf [29]. Net gas exchange fluxes can help constrain the labeling kinetics of metabolite pools to map fluxes through central carbon metabolism [30]. A similar approach allows for a detailed flux analysis of specific biochemical pathways such as photorespiration, which has been achieved with leaf-level gas exchange in combination with either modeling [31] or quantitative NMR analysis [32].

Often, leaves are not the only photosynthesizing organs and other non-foliar tissues may significantly contribute to overall plant photosynthetic carbon gain [33]. Of particular note here are stems and fruit [34], ears and panicles [35, 36], and pods [37]. Measuring non-foliar photosynthesis follows similar principles of that in leaf tissues but usually requires some specialized chamber, as described herein [38].

CO_2 exchange can also be measured in aquatic organisms, ranging from unicellular and macro-algae to aquatic plants and

even corals. Measurements of this type in liquid are more complicated than measurements on leaves surrounded by air due to the slower acclimation time when changing environmental conditions and needed assumptions regarding the solubility of CO_2, which is dependent, for example, on the pH of the liquid. Currently, only one commercial product exists that allows for relatively straightforward measurements of CO_2 exchange in liquid. Details about this specialized chamber and how to successfully obtain measurements are described herein [39].

2.2 Whole-Plant CO_2 Exchange

Leaf-level gas exchange, as described above, can yield valuable insights into the physiology and biochemistry of the leaf. However, in some cases, these measurements cannot be obtained directly, e.g., when the leaves are too small or oddly shaped to be measured via a clamp-on leaf chamber. In addition, for some purposes, leaf-level measurements are too specific, as they only include measurements of the photosynthetic tissue of the leaf and neglect the effect of other parts of the plant that are not contained within the chamber, such as other leaves, stems, and roots. This is particularly an issue when one wants to relate photosynthetic rate to plant growth or to integrate photosynthesis across leaves of different ages on the same plant. One way around these issues is to measure the CO_2 uptake of the enclosed shoots of an entire plant [40].

Whole-shoot measurements of plant CO_2 exchange also provide different types of data as compared to leaf-level measurements. For example, the CO_2 uptake integrated over the entirety of the plant and the whole growth period can be used to estimate growth nondestructively in real time through carbon balance when it is measured at regular enough intervals [41]. In addition, whole-plant experiments can be combined with carbon isotope labeling to obtain insights into carbohydrate metabolism [42]. The benefit of a whole-plant approach in labeling approaches is that more than one leaf of the plant is labeled, allowing the inclusion of the effects of both photosynthetic and non-photosynthetic tissue of the above-ground parts of the plant in experimental analyses. Whole-plant exchange can be measured in much the same way as leaf-level exchange by means of closed, open, or semi-closed chambers placed over or around the plant to be measured. Gas exchange is measured by the drawdown of CO_2 within the chamber for closed and semi-closed systems, or the difference in CO_2 and H_2O concentration between the air entering and exiting the chamber in open systems. Chamber-based measurements can be effectively employed but are biased by differences in temperature, light intensity, turbulent mixing, and CO_2 concentration between the inside and outside of the chamber (see [43]). Many of these differences can be minimized through the construction of semi-closed systems with air-conditioners, mixing fans, dehumidifiers, and CO_2 injection systems [44]. Chamber-based methods can be deployed on the

scale of small herbaceous plants [45], and recent developments in multi-channel systems enable the measurement of intact shoot material in many plants simultaneously [40]. Chamber-based measurements have even been scaled to fully-grown plants such as in the Hawkesbury Forest experiment where *Eucalyptus salinga* trees are reared from seedlings and grown within chambers that allow for trees up to 9 m in height [46]. The Hawkesbury Forest chambers are revealing important physiology in relation to climate change; for example, gas exchange data from chambers with elevated temperatures revealed that in a *Eucalyptus* species, the relative increase with temperature is larger for respiration than for net photosynthesis despite physiological adaptation to growth conditions, which may exacerbate the impacts of climate change in these species since their net CO_2 uptake will decrease with temperature [47].

Some techniques that work well on the leaf level cause problems on the whole plant level; whole-plant gas exchange measurements are difficult when performing responses to environmental conditions, such as temperature and light, since these variables are not easily controlled uniformly across the whole canopy of the plant. An additional consideration is the increased investment in equipment, reagents, and setup time, especially since there is currently a lack of commercially available instruments and most systems must be custom-built.

2.3 Canopy CO_2 Exchange

The next step along the continuum of scales after whole-plant measurements is the measurement of CO_2 exchange at the canopy scale. Canopy-scale measurements of CO_2 and H_2O exchange are most useful when research questions are focused on the interaction between plants and the growth environment, for example, to determine the net carbon balance of ecosystems in response to present and future climates [48]. Canopy photosynthesis is measured in much the same way as whole plant measurements by enclosing a portion of the canopy in a translucent chamber and measuring gas fluxes either via an open, closed, or semi-closed path design. Canopy chambers are helping to resolve the importance of canopy effects on in-field photosynthesis, and recent advances in automation have made them more practical for larger-scale studies as discussed herein [49].

Eddy covariance has become a powerful method to determine CO_2 and H_2O exchanges from canopies ranging in size from hundreds to thousands of meters non-invasively and over long time scales [48, 50]. Eddy covariance measures flux into and from the canopy by measuring trace gas (CO_2 and H_2O) concentrations in tandem with wind speed and direction. These data are analyzed using a statistical model that represents turbulent mixing to produce measurements of canopy CO_2 and H_2O exchange. This technique has been employed in a myriad of sites around the world and is more straightforward over "smooth" vegetation like most

agricultural systems, but more complicated over "rough" canopies such as those found over forests [51, 52]. An introduction to the eddy covariance technique and how it is applied to measure photosynthesis is included herein [53].

2.4 Measurements of O_2 Exchange

The O_2 produced during H_2O splitting from photosystem II (PSII) provides a direct assay of the activities of the light reactions. O_2 in living systems was first measured using manometric techniques before a critical review of cardiovascular researcher Leland Clark's work with blood oxygenation led him to develop an electrode for the measurement of dissolved O_2 [54]. The Clark-type electrode determines dissolved O_2 concentration by monitoring the reduction of O_2 catalyzed via a platinum surface separated from the liquid being assayed by a semipermeable membrane [55]. O_2 electrodes are routinely used to determine the photosynthetic capacity of algal cultures and chloroplast suspensions and have even been adapted for use with excised leaf disks. Rates are determined by monitoring the increase of dissolved O_2 as a function of time in a closed, illuminated reaction cuvette. O_2 electrodes can also be employed for gas-phase measurements, but for greater accuracy, on-line mass spectroscopy can be employed as discussed below and within this book, with techniques and background on both approaches presented herein [56–58]. Measurements of O_2 exchange in algal cultures and isolated chloroplasts were instrumental in resolving the maximum quantum efficiency of photosynthesis (discussed further below), which was critical for subsequent work exploring its mechanisms [59]. Measurements of O_2 exchange are still valuable alone but are especially informative when combined with isotopic methods as discussed below.

2.5 Using Isotopes to Resolve Net Fluxes of Gas and Physiology

While measurements of the molecular fluxes of O_2, CO_2, and H_2O vapor provide a rich source of information on the photosynthetic physiology of the measured sample, parallel analysis of stable isotopes performed on the same fluxes can be used both to expand and better constrain the analysis as discussed herein [28]. Small predictable differences in reaction and diffusion rates between lighter and heavier isotopologues of CO_2 and H_2O create small alterations in the natural abundance of stable isotopes during photosynthetic gas exchange. Slight changes in the relative abundance of $^{13}CO_2$ can be used to quantify internal conductance to CO_2 in C_3 species [60–62], establish the presence and expression of carbon concentrating mechanisms [63], as well as the extent of CO_2 leakage away from the site of concentration [64]. Simultaneous determination of changes in the relative natural abundance of ^{18}O in CO_2 and H_2O vapor can be used to determine the internal conductance to CO_2 in C_4 species and the CO_2 permeability of the chloroplast membrane in C_3 species [65]. These techniques make use of changes in the natural abundance of stable isotopes. However, it

is also possible to manipulate the stable isotope composition of air to directly distinguish between in- and outgoing gas fluxes of leaves. For example, fluxes of O_2 evolution and O_2 uptake in leaves can be determined using air heavily enriched in $^{18}O_2$ and $H_2{}^{18}O$ [66, 67]. Similarly, when using air heavily enriched in $^{13}CO_2$, distinction can be made between, e.g., fluxes of respired ($^{12}CO_2$) and assimilated $^{13}CO_2$ [68]. These flux measurements can also provide an estimation of the chloroplastic CO_2 concentration and, thereby, can be used to quantify the internal conductance to CO_2 between the intercellular air space and the chloroplast [29]. These isotope exchange techniques are often combined with chlorophyll fluorescence measurements [69–71]. When using isotopologues of CO_2 with singly ($C^{18}O^{16}O$) and doubly labeled O_2 ($C^{18}O^{16}O$), it is possible to study specific enzyme activity in vivo of carbonic anhydrase in leaves [72]. These types of analyses allow relatively fast kinetic studies in vivo (seconds and minutes), providing a more detailed dissection of processes underlying CO_2 and O_2 gas exchange of leaves. Moreover, when using air heavily enriched with isotopologues for prolonged periods (minutes, hours, and days), isotopic labeling of metabolites, proteins, and structural components can be achieved in so-called pulse-chase experiments, such as described herein [73]. This longer-term isotope labeling, especially when combined with leaf gas exchange techniques, can provide valuable insight into metabolic pathways, fluxes, and enzyme activities and changes therein as discussed above.

3 Light-Based Probes of Photosynthesis

The unique photochemistry of the light reactions provides several useful tools for understanding photosynthetic rates from the perspective of light energy utilization and subsequent electron transport (Fig. 3). As discussed below, these light-based probes of photosynthesis are non-invasive and can be applied remotely to resolve the flux of electrons and protons in vivo through photosynthetic systems. These techniques differ from gas exchange methods in that flux is not measured directly via the uptake of substrates but rather via the interactions of molecules and proteins with photochemically-driven redox reactions or protonation states. These signatures of photosynthesis are then detected as emitted photons, as is the case with chlorophyll fluorescence, or as shifts in light absorption at characteristic wavelengths. The emitted photons and/or absorbance shifts are then used to derive either operational efficiencies in the case of chlorophyll fluorescence and PSI redox state or relative flux units as is the case with the electrochromic shift (ECS). As with gas exchange, these techniques can be applied from

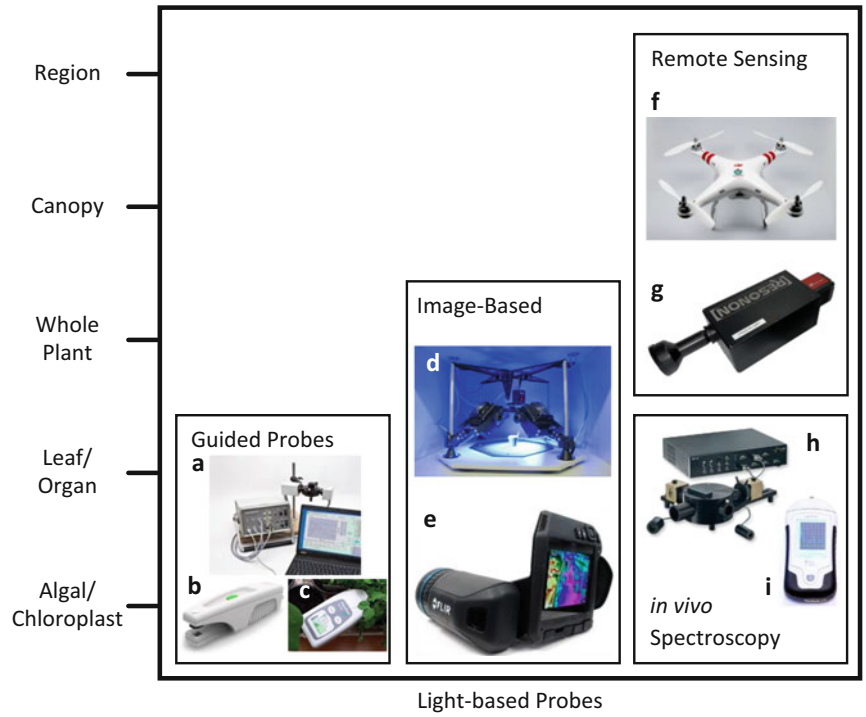

Fig. 3 Tools used to assay light-based biophysical probes of the light reactions across diverse scales. Shown are Pulse Amplitude Modulated (PAM) Fluorometry methods including "guided probe" approaches, which delivers measuring and actinic light via fiber optic cables or LED sources in close proximity to the leaf surface, such as the Dual-PAM-100 from Heinz Walz GmbH (**a**), the MultispeQ from PhotosynQ (**b**), and the FluorPen from Photon Systems Instruments (**c**) as well as imaging-based platforms such as the Open FluorCam from Photon Systems Instruments (**d**). Thermal images of leaves or whole plants can be obtained with IR cameras such as the FLIR T540 from Teledyne FLIR (**e**). Remote sensing platforms include the use of drones such as the Phantom series from Dà-Jiāng Innovations Science and Technology Co (**f**). Remote multispectral imaging is possible with devices such as the Resonon Pika series cameras (**g**). Monitoring of other spectral signatures can be accomplished using the JTS-10 spectrometer from BioLogic Science Instruments (**h**) and the SpectraPen from Photon Systems Instruments (**i**). Instruments from the various manufacturers are shown only for informational purposes and do not imply any specific recommendation. The above images are reproduced with kind permission from their respective copyright holders: Heinz Walz GmbH (**a**), PhotosynQ (**b**), PSI (Photon Systems Instruments) spol. s r.o. (**c**, **d** and **i**), Mengjie Fan and Tracy Lawson (**e**), Clément Bucco-Lechat under a Creative Commons Attribution-Share Alike 3.0 license (**f**), Taylor Pederson (**g**), and BioLogic Science Instruments (**h**)

the single leaf level to entire canopies and, to some extent, monitored remotely using drone or satellite imagery.

3.1 Chlorophyll Fluorescence

Chlorophyll fluorescence is a powerful tool for probing the operation of photochemistry at the level of PSII, the enzyme complex responsible for H_2O splitting during photosynthesis, and providing electrons for downstream photochemical energy conversion from absorbed light energy [74–76]. The link between chlorophyll fluorescence and photochemistry lies in the various fates of light energy

absorbed by a chlorophyll molecule. Once excited, there are three main routes by which absorbed light energy is "quenched," or dissipated [77]: (1) photochemical quenching through the passage of the excitation energy to PSII, where it is used to transfer electrons from H_2O to the mobile electron-carrier plastoquinone, (2) non-photochemical quenching when the excitation energy is dissipated as heat, and (3) chlorophyll fluorescence when the energy is re-emitted as a photon with a shifted wavelength. Since chlorophyll fluorescence represents the balance of the three processes and is readily measured, it can be monitored under various conditions to understand relative rates of photochemical and non-photochemical quenching [78]. Fluorescence can be measured using "guided probe" systems, which orient the measuring and excitation beams close to the leaf surface with an optic fiber or via more remote tools such as imaging systems (as described below) or more recently via solar-induced fluorescence measured from towers or using satellites [79].

The deconvolution of quenching fates is accomplished by measuring chlorophyll fluorescence emitted before and during a short (<1 sec) flash of light that saturates the capacity of photochemical quenching resulting in a proportional increase in fluorescence [80]. The transient rise in fluorescence can then be used to infer the behavior of photochemical quenching during the condition immediately before the saturating flash of light. One difficulty with this approach is imposing a flash of light that fully saturates PSII without over-saturating PSII and inducing alterative dissipative fates for absorbed light energy. While this can be accomplished by careful selection of saturating light intensities, the problem can also be circumvented by exposing the leaf to a multi-phase flash of sub-saturating intensities and extrapolating fluorescence yields to a saturating value [81]. The pre-flash condition can either be a light or dark-adapted state, and each reveals details of leaf light use in the presence or absence of non-photochemical quenching, respectively. Chlorophyll fluorescence can be measured on "fast" or "slow" timescales to reveal potentially complementary information on the fate of absorbed light energy. "Fast" timescale measurements (millisecond) attempt to deconvolute the transient induction of the fluorescence signal in response to a saturating light flash into various phases of PSII electron transfer of a leaf in the fully dark-adapted state [82]. Techniques in making sensible "fast" fluorescence measurements are discussed herein [83].

Chlorophyll fluorescence is often used at the "slow" time scales to resolve details of photochemical and non-photochemical quenching. In this chapter, we refer to "slow" timescales as looking at the maximum fluorescence yield during the application of a saturating light pulse without resolving the kinetics of that initial induction as mentioned in the previous paragraph. These measurements can be used in both the light and dark-adapted state, to

resolve actual rates of linear electron flux, non-photochemical quenching, and the maximum efficiency of PSII [75, 76]. These data are also important for determining the induction or relaxation kinetics of photochemical and/or non-photochemical processes, particularly in combination with changes in environmental variables such as temperature, light, or CO_2 [84]. Changes in these estimations and kinetics can function as a non-invasive proxy for a plant's stress responses because of light-induced damage to the photosynthetic apparatus [85–87]. Recently developed techniques in using sub-saturating flashes of light are making these measurements even less invasive with techniques discussed herein [88]. Leaf-level chlorophyll fluorescence can also be combined with CO_2 and O_2 gas exchange to determine energy partitioning between CO_2 assimilation, photorespiration, respiration, and other processes [70, 71].

Leaf-level chlorophyll fluorescence measurements have been applied broadly to probe and predict many aspects of plant physiology. For example, the CO_2 fixation rate of C_4 plants can be accurately determined through a now-simple measurement of quantum efficiency using chlorophyll fluorescence since the majority of reductant in this photosynthetic type goes toward carbon reduction [89]. Furthermore, since the non-photochemical quantum yield is related to other plant stresses, dark-adapted chlorophyll fluorescence measurements have been used to screen for cold hardiness in barley [90] and other crops.

3.2 Chlorophyll Fluorescence Imaging

Chlorophyll fluorescence imaging provides the opportunity to spatially resolve chlorophyll fluorescence signals and parameters at a range of different scales. High-resolution chlorophyll fluorescence microscopes enable photosynthetic efficiency (and the associated quenching parameter) to be determined at the cellular [91, 92] and the sub-cellular [93] levels, while the integration of chlorophyll fluorescence into many large-scale industrial and commercial phenotyping platforms has provided the ability to screen large numbers of plants. Such capabilities have been invaluable for screening for changes in plant metabolism, for example, improvements in photosynthesis [94] and the impact of herbicides [95]. Many of the commercial instruments can be used on a range of different photosynthetic materials—from screening algae in media in Petri dishes [96] to plants in 96-well plates [95], from individual leaves [97] to whole plants [98]. Chlorophyll fluorescence imaging also allows spatial heterogeneity to be assessed, which is often present within or between samples [99]. This can be a problem with traditional fiber optic approaches that only measure a small proportion of the photosynthetic material defined by the users, which can lead to increased variation between measurements due to spatial heterogeneity. Chlorophyll fluorescence imaging can also be combined with other physiological techniques to provide advanced screening methodologies and powerful physiological measurement

approaches. For example, combined chlorophyll fluorescence and thermal imaging have been used to screen for intrinsic H_2O use efficiency [100], and chlorophyll fluorescence imaging in combination with Infra-red gas exchange has been used to produce spatial images of internal CO_2 concentrations within the leaf in order to calculate lateral gas fluxes [101]. Carrying out chlorophyll fluorescence imaging under known gas environments has also been used as a screening tool for photorespiratory mutants [102].

The advantages of chlorophyll fluorescence imaging have been outlined above and are explored in greater detail herein [103]. However, disadvantages include the size of the equipment, which often means that such imaging systems are not portable and often rely on mains power. There are exceptions to this, with portable field-based systems commercially available, although the area for imaging is, in general, small compared to many of the lab instruments. Another challenge is that due to the large imaging area of many systems, it can be difficult to obtain even actinic illumination over the entire imaging area, which can result in the introduction of spatial variation into the images. Another problem with illumination can be the intensity of the saturating pulse, which can be lower than 4000 μmol m^{-2} s^{-1} and is, therefore, unlikely to be saturated for species grown in high-light environments or that perform C_4 photosynthesis.

3.3 Thermal Imaging

Another dimension of the leaf-to-canopy-level information can be drawn from thermal imaging. Highly sensitive cameras are available now that resolve leaf or canopy temperature remotely and in detail, allowing for spatial or temporal visualization of processes related to transpiration. Evaporating water that is transpired through the stomata of a leaf carries heat away, which results in the cooling of the leaf. Leaf temperatures, when taken in comparison to that of reference surfaces, can thus indicate the rate of transpiration and associated stomatal conductance [104]. These "wet" and "dry" reference temperature measurements are crucial for accurately determining stomatal conductance but also can make thermal imaging challenging to perform in variable outdoor environments. Alternative approaches based on calculated energy balance have made it somewhat easier in recent years to obtain reliable measurements [105, 106]. How to best use thermal imaging in connection with high-throughput phenotyping is described herein [107]. Because of the complementary information that can be drawn from thermal imaging, it is often used in connection with chlorophyll fluorescence imaging to obtain spatially resolved information on stomatal opening and photosynthetic activity [108].

3.4 Whole-Leaf Spectroscopy

Characteristic absorbance shifts originating from components of electron transport downstream of PSII provide valuable insight into the fate and results of light energy capture. The two main spectral signatures downstream of PSII stem from the reaction center chlorophyll pigments of photosystem I (PSI), P700, and from the ECS of carotenoid proteins embedded in the thylakoid membranes [109]. The redox status of P700 is estimated by absorbance shifts in the 800–850 nm range as measured under steady state and rapid-saturating light pulses, thus probing the quantum efficiency of PSI [82, 110]. Since the ECS responds to the electric field across the thylakoid membrane, the relaxation of the ECS in a dark interval is proportional to the proton motive force maintained by proton pumping and its decay kinetics provide information concerning the conductivity of ATP synthase [111, 112].

The absorbance shifts driven by P700 and the ECS can be measured on an intact leaf and are helping to resolve important aspects of how the light reactions flexibly provide reductant and ATP to optimally provide for the dynamic demands of carbon fixation, photorespiration, and other aspects of central metabolism without resulting in the damaging consequences of over-reduction of the electron transport chain [113, 114]. These spectral signatures have been used to provide indications of cyclic electron transport around PSI when measured in conjunction with PSII chlorophyll fluorescence [115, 116], determine the light response of PSI quantum efficiency as affected by environmental variables [117, 118], and estimate in vivo steady-state proton fluxes, ATP synthase activity, and components of the transthylakoid proton motive force [115, 119, 120].

The above-mentioned changes in leaf absorbance are related to known mechanisms at the physiological or biochemical level that cause these changes. However, it is not always necessary to know the underlying mechanisms and much can be learned through empirical correlation of measured absorbance changes with underlying changes in biochemistry. Changes in photosynthetic metabolism often manifest with an imprint onto leaf optical properties, which can be measured over a wide range of the electromagnetic spectrum using reflectance spectroscopy. Partial least-squares regression analyses can be used to sift through the large amount of data obtained and filter out the key determinants of the photosynthetic property of interest, such as V_{cmax} or J_{max} [121]. This approach allows for rapid phenotyping of plants to get spectroscopic proxies for these parameters without having to perform the detailed measurements that would be necessary to characterize the plants biochemically [122]. Spectral reflectance data may be obtained from point measurements on individual leaves, or can be measured by multispectral (~ 3–15 targeted spectral bands) or hyperspectral (often 100 s of continuous spectral bands) imaging on scales from the leaf to the canopy [123, 124]. Depending on the

equipment used, a variety of parameters relevant to photosynthesis can be estimated, from pigment content and status of the xanthophyll cycle to photosynthetic capacities. Detailed guides on considerations and practices for equipment setup and data collection are described herein [125, 126]. Best practices for the analysis of hyperspectral imaging data are also discussed in this book [127].

3.5 Light Response Curves

Measurements for probing photosynthesis, such as gaseous exchange of CO_2 or O_2, chlorophyll fluorescence, or wavelength-specific absorption changes, are often integrated into experiments where environmental factors such as CO_2, temperature, or light intensity can be varied. In the case of light intensity, several important properties can be extracted from such measurements as discussed herein [18].

Saturated photosynthetic capacity can be derived as the asymptote to which the response curve saturates at infinite light intensity. Additionally, the initial slope of the response provides a measure for the quantum efficiency, i.e., the maximum number of evolved O_2 molecules or assimilated CO_2 molecules per absorbed photon. This parameter is typically designated by the symbol φ and was instrumental in investigating a long-running scientific controversy concerning the photon requirements of water splitting (reviewed by [59]) until the involvement of two photosystems in photosynthesis was discovered [128] and the development of the Z-scheme [129] provided firm theoretical underpinning for the minimum requirement of eight photons per evolved O_2.

While in the 1950s photosynthetic light response measurements were performed using extremely tedious and error-prone manometry, modern instruments utilize more user-friendly techniques to measure the light response of photosynthesis via liquid or gas phase gas exchange. It is however still important to realize that the measurement conditions during the light response curve (temperature, light spectrum, atmospheric gas composition, etc.) as well as pre-treatment of the sample can have important consequences for the measured light response. Photoinhibition or non-photochemical quenching can substantially reduce the quantum efficiency of photosynthesis (φ, [130]) and induce substantial variation in measured values, whereas φ measurements on non-stressed, fully dark-adapted material typically lead to a very narrow range of values [131]. Considering the lack of variation associated with dark-adapted φ in C_3 photosynthesis, differences in the photosynthetic light response can reflect physiological adaptations of photosynthesis, such as the presence or absence of a carbon-concentrating mechanism (e.g., [132]). For instance, in species with C_4 photosynthesis, the additional 2 ATP required for the carbon-concentrating C_4 acid shuttle is reflected in φ, and light saturation of photosynthesis also typically occurs at considerably higher light levels compared to C_3.

Light response curves are deceptively tricky to execute properly. For example, in the case of leaf gas exchange, it can be challenging to keep leaf temperature, CO_2 concentration, and vapor pressure deficit constant with variations in light intensity. In traditional light response curves with sequential changes in light intensity on the same sample, it is also important to consider the order of changes in light intensity. A measurement sequence from high to low light intensity helps to fully induce enzyme activity and stomatal opening at the start but will also generate substantial non-photochemical quenching or even photoinhibition, leading to decreased φ. Alternatively, measurements from low to high light intensity can provide a non-biased estimate for φ but require much more time to allow full enzyme activation and stomatal opening after each increase in light intensity and rushed measurements would again lead to underestimation of φ, especially in the intermediate light range. Both examples emphasize the issue of performing measurements at different light intensities sequentially on the same sample, which carries the risk of generating a light history component throughout the response curve. Subdividing the sample to measure light intensities in parallel can be used to avoid this problem, as was demonstrated by [133]. This method also holds promise to reduce the time required per response curve from approximately 0.5–1 h to only a few minutes; however, its interpretation is limited to values derived from chlorophyll fluorescence since it is not able to resolve leaf gas exchange.

4 The Future of In Vivo Measurements of Photosynthetic Flux

The future of measurements of photosynthetic flux relies on research that exploits the unprecedented accessibility of current technology to multiplex and increase the throughput of measurements at diverse scales. As discussed below, these developments will provide novel insight into basic plant biology and help bring photosynthetic insights into -omic scale approaches.

The ability to multiplex, or perform simultaneous assays on the same sample, is a hallmark of genomic and metabolomic-based approaches and have helped to greatly advance these fields and holds promise to help advance our understanding of photosynthesis. Given the inherently lower-throughput limitations of photosynthetic measurements that require physically clamping a measuring device to each leaf, it makes sense to combine many assays in the same device to get as much data from each measurement as possible. Additionally, deeper physiological meaning can be determined when certain measurements are combined, for example the determination of the CO_2 transfer conductance across the mesophyll from combined measurements of gas exchange and chlorophyll fluorescence [134]. Past efforts in multiplexing

photosynthesis measurements have been limited by the physical size of each component, but recent advances in LED technology and market pressure to engineer more compact analytical equipment has helped alleviate this problem. For example, a recently launched effort (PhotosynQ) to provide a low-cost, open-source instrument has produced a hand-held device capable of measuring over a dozen different signatures of photosynthesis during a 15 second measurement [135]. The PhotosynQ platform additionally uploads time-stamped and geotagged data into a freely accessible database, allowing leaf-level measurements to be interpreted at multiple scales. Another example in multiplexing is when measurements of carbon assimilation have been combined with mass flux balance approaches to constrain the photosynthetic uptake of carbon in models of central metabolism [30] and for confirming the stoichiometry of CO_2 release from photorespiration [32].

In the past, the limited throughput of tools measuring photosynthetic flux has restricted their application from approaches tying gene to function, such as through parallel quantitative trait locus mapping and genome wide association studies [136, 137]. Increasing measurement throughput is also important to properly screen transgenic plants carrying multi-gene constructs for increased photosynthesis in replicated field trials. Measuring in vivo photosynthetic parameters in tandem with other techniques, e.g., including those discussed herein such as extracting soluble proteins from leaves [138], quantifying photosynthesis-related enzymes with antibodies [139], purifying Rubisco for biochemical applications [140], evaluating thylakoid lipid content [141] and stable isotope labeling for the quantification of metabolites [73], would be useful to more fully understand the physiological and molecular effects of gene function or environmental changes, as determined by the experiment. Fortunately, there are several emergent approaches that may help increase throughput, in addition to the PhotosynQ platform mentioned previously. For example, key photosynthetic parameters derived from measurements of the response of carbon assimilation to CO_2 (maximum rate of Rubisco carboxylation and electron transport) correlate strongly with spectral regions of the fresh leaf hyperspectral reflectance [121, 142]. Since hyperspectral leaf reflectance measurements take <1 s as compared to 20–40 min for the standard measurement by gas exchange, this approach holds great promise for application for higher throughput phenotyping (see, e.g., [122]), yet the mechanism behind this relationship remains unresolved. Validation of this approach, and of determining photosynthetic gas exchange parameters in general from gas exchange, will be aided by the development of non-steady-state approaches for measuring the response of photosynthetic assimilation to CO_2 concentrations [143, 144].

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