Study of 660 nm laser-induced photoluminescence of chlorophyll-a and its applications

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Abstract. Based on the phenomenon of chlorophyll a photoluminescence, this paper introduces a new method to measure the chlorophyll a content, using 660nm laser diode as a new kind of light source to stimulate fluorescence as well as combining a fiber and spectrum technique. We analyze the characteristics of laser-induced fluorescence spectrum of chlorophyll a and then put forward the new method using two parameters, the relative fluorescence intensity and fluorescence intensity ratio F685/F735, to measure the chlorophyll a content in the water and green leaves respectively. The experimental results indicate that it is completely feasible to give a visual judgment for chlorophyll a content according to the fluorescence emission spectrum of chlorophyll a. Subsequently, it is verified by three kinds of typical applications. All of these provide a new kind of light source to develop the chlorophyll a fluorometry and further give a technical foundation of on-spot monitoring the chlorophyll a content in the ocean or in green leaves.

Keywords: photoluminescence, chlorophyll a content, chlorophyll a fluorescence emission spectrum, fluorescence intensity ratio F685/F735

1. Introduction
Chlorophyll a is an activator of the photosynthesis of plants. Chlorophyll a content in the water that determined the amount of phytoplankton is one of the most important indices to monitor the water quality of the ocean, it also can estimate the primary productivity in the ocean with the chlorophyll a content [1]. Consequently, it can forecast the appearance of the red tide [2]. Moreover, Chlorophyll a content in plants, which can represent the photosynthetic activity of green plants, is a significant parameter to monitor the growing state of plants [3]. Therefore, it is much important to measure the chlorophyll a content.

For a long time, the usual methods to measure the concentration of chlorophyll a are the spectrophotometry and fluorometry [4-6]. The former cannot be applied on real-time measurement due to the poor sensitivity, the long measurement cycle and the disturbance of other pigment. So the researchers now often use fluorometry to measure the chlorophyll a content in the water. But for the measurement of the chlorophyll a content of plants there is almost not any new methods to be mentioned and the spectrophotometry is still be used. Moreover, as the chlorophyll fluorescence signal is very weak it usually needs an excited light with high strength, no disturbance of background noise and the steady output. Although several instrumentation use monochromatic light, such as bright LED arrays of 650 nm, a 635nm laser diode[7], but most researchers still use the traditional multicolored light as the excited light (include Hg-lamp, Xe-lamp, all kinds of plasma light source and so on). It needs to make some extra measurements on filtering the light, magnifying the signal, removing the noise, etc. Due to these, the system undoubtedly will become more complicated and do harm for the real-time measurement. Contrastingly, because laser has high strength and narrow pulse width, the sensitivity of measurement by using laser-induced fluorescence commonly is 2-10 times than using other light source. Although some researchers from home and abroad have used laser as the excited
light, but used to use blue or green laser with high energy waste and expensive cost as well as the gas laser with big volume [8]. Furthermore, a violet laser diode (LD) has also been used to illuminate the chlorophyll a fluorescence, but it may induce blue fluorescence as an undesired signal, some obscure fluorescence parameters and its applications are limited due to its lower penetrating capacity and higher cost. This paper introduces the fluorescence intensity ratio of F685/F735 as a parameter to evaluate chlorophyll a content of green plants. A new method to make a fast measurement on the chlorophyll a content of green plants was further developed. We first use the red laser diode with low energy consumption as excited light combining a Fiber-optic spectrometer to enhance the ability of collecting the fluorescence signal, due to these improvements, the final system will be high sensitivity, low energy waste and miniaturization.

2. Principle and method

2.1. The photoluminescence mechanism of chlorophyll a
When irradiated by the light with certain wavelength, the color of chlorophyll a solution seems green in the incidence light, but seems red in the reflection light, this phenomenon is called chlorophyll a photoluminescence (fluorescence). Because after the chlorophyll a molecule partly or entirely absorbs the photons whose frequency equals the inherent frequency of chlorophyll a, it rises from the most stable and lowest energy state-based state to the unstable and high energy state-excited state and then the molecules on different vibration ranks of certain excited state, will first return to the lowest vibration rank of the first excited state in a short time (10^{-12}s) by no radiations, and then return to the different vibration ranks of the base state by radiations(10^{-8}s), eradiating the fluorescence [9].

2.2. The characteristic of fluorescence emission spectrum of chlorophyll a
Because the energy of light absorbed partly depends on the vibration of the molecule, so the wavelength of the fluorescence is always longer than the wavelength of excited light. It can be visually described by the fluorescence emission spectrum of chlorophyll a. The laser-induced photoluminescence (or fluorescence) intensity only depends on the chlorophyll a content as long as the output intensity of the excited light is steady. For the fluorescence emission spectrum of chlorophyll a solution, the general rule is that there is an obvious fluorescence peak around 685 nm, and the higher the relative fluorescence intensity (685 nm), the higher the content of chlorophyll a in the solution, they are of approximately linear relation to each other when the solution is thin enough. The Lambert-Beer Law can derive the specific expression [10]. Suppose the incidence light with intensity of I_0 vertically irradiates in the chlorophyll a solution, By the Lambert-Beer Law, when the solution is thin enough, the fluorescence intensity $I_f$ is:

$$I_f = (\varphi_f I_0 e^l \ln 10) c = K c$$  \hspace{1cm} (1)

From Eq.1: $\varepsilon$ is the Moore absorbed coefficient, $c$ is the concentration of chlorophyll a solution, $l$ is the depth of the solution, $\varphi_f$ is the fluorescence efficiency. Obviously, when parameters of equipments are assured, $K$ is a constant.

Eq.1 is a fundamental of the fluorometry, we can infer from this equation that the fluorescence intensity is in proportion to the intensity of fluorescence substance. The fluorescence emission spectrum of chlorophyll a in green leaves (induced by the excited light with wavelength>360nm), however, is quite different from that in the solution. Firstly, the fluorescence emission spectrum of chlorophyll a in one leaf has two maxima near 685 nm and 735 nm. Secondly, research shows that the chlorophyll absorption band and the fluorescence emission band overlap around 685nm region, for the very high chlorophyll a content in green leaves, the 685nm fluorescence peak decreases with chlorophyll a content increases, due to preferential reabsorption of the emitted 685 nm fluorescence[11-13]. In this case, the single 685nm fluorescence intensity can no longer be used to
measure chlorophyll a content. Therefore, we propose a new method to measure chlorophyll a content with the fluorescence intensity ratio of F685/F735, for it is mainly determined by the chlorophyll a content of the leaf. We will also conclude the specified relation between the ratio F685/F735 and the chlorophyll content of the green leaf in this article.

2.3. Method and equipments
In the experiment we use laser-induced fluorescence as well as combining a fiber and spectrum technique to measure the fluorescence emission spectrum of chlorophyll a in the water and in green leaves separately. The steps are given as follows: first, we use red laser diode to stimulate the fluorescence of samples, and then use the Fiber-optic spectrometer to get the fluorescence emission spectrums and show them on the computer synchronously, finally, we accurately work out the 685nm fluorescence intensity and the fluorescence intensity ratio F685/F735 with the software we compiled. Consequently, we can get a fast and visual judgment about the chlorophyll a content from the spectrums. Primary instruments used in the experiments are: HR-2000 Series Fiber-optic spectrometer and appendix, red laser diode (660nm) with output power 10 mw (produced by Changchun New Industries Optoelectronics Tech. Co., Ltd.), 722-visible spectrophotometer produced by Shanghai Changfang Optical Instrument Co., Ltd.), SL-502 electronic balance (500g/0.01g, produced by Beijing UESIPHY Development Co., Ltd), narrow band-pass filter (685nm center wavelength, 15nm bandwidth and 45% peak transmittance, produced by HB Optical Co., Ltd.), computer, splitter (1cm x 1cm x 1cm), experimental desk, pure ethanol (95%), graduated cylinder (25ml), vessel, etc. The schematic diagram of the equipment is shown in Figure 1-1, 1-2.

3. Applications
3.1. Measure the concentration of chlorophyll a in the solution
With the above principle and method we can demonstrate the relation between chlorophyll a content in the solution and 685nm fluorescence intensity, then create a new method to measure the concentration of chlorophyll a solution fast and visually. The details are: we first pick some fresh leaves, mix with high purity ethanol (95%) 5ml to the standard solution after cutting and grinding, assume its concentration is \( c_0 \), and then get its fluorescence emission spectrum, assume the relative fluorescence intensity is \( F_0 \), next, gradually dilute the standard solution to 6ml, 8ml, 10ml, assume the concentration of these three solutions is \( 5c_0/6, 5c_0/8 \) and \( c_0/2 \), then measure the corresponding fluorescence emission spectrum of different concentration solutions on the same conditions with the equipment shown in Figure 1-1, assume the corresponding fluorescence intensity is \( F_1, F_2 \) and \( F_3 \), the spectrum and some experimental data are shown in Figure 2,3.

In Figure 2 we can find that the fluorescence emission spectrums of these four chlorophyll a solutions all show an apparent 685nm fluorescence peak, they are of approximately linear relation to each other, and it is shown in Figure 3. The corresponding regression equation concluded by the least squares method in Figure 3 is: \( y = 183.1 \times x + 22.68 \), the correlation coefficient is 0.995. In this case, as long as we know the concentration \( c_0 \) of the standard solution (or measure it by spectrophotometry), we can compute the concentration \( c_x \) of the unknown solution according to the ratio of 685 nm fluorescence intensity \( F_x \) (unknown solution) and \( F_0 \) (standard solution), namely, \( c_x = c_0 \times F_x / F_0 \).

3.2. Evaluate the chlorophyll content in green leaves (bamboo-leaf and tea)
With the above principle and method we also can evaluate the chlorophyll content in green leaves, further can monitor the growing state of plants and the vitality of the photosynthesis. The details are: firstly, pick four different maturity of fresh bamboo-leaves (Mar) and tea-leaves (Mar) from the same branch, cut and weigh 1g leaf from the same extent of every kinds of leaves, and divide them into two parts with same weight. Secondly, we use spectrophotometry to measure the chlorophyll content of 0.5g leaf (The chlorophyll content is measured from the high purity ethanol extractive solution with
the volume of 50ml, the details can refer the fourteenth citation) [14], assume the content of these four leaves is $C_1, C_2, C_3, C_4$, in increasing order. Thirdly, with the equipment shown in Figure 1-2, we can get the fluorescence intensity ratio $F_{685}/F_{735}$ which is measured at maximum fluorescence by using red LD to irradiate another half of these four leaves separately, assume the corresponding fluorescence intensity ratio is $R_1, R_2, R_3, R_4$, and then make linear regression to get the relation between chlorophyll content and the ratio of $F_{685}/F_{735}$, they are shown in Figure 4, 5.

Generally, we can see from Figure 4, 5 that the higher the chlorophyll content, the lower the fluorescence intensity ratio $F_{685}/F_{735}$, and they are of approximately linear relation to each other. Specifically, the corresponding regression equation concluded by the least squares method in Figure 4 is: $y=-0.53 \times x + 2.31$, the correlation coefficient is 0.998. Similarly, the corresponding regression equation in Figure 5 is: $y=-0.72 \times x + 2.64$, the correlation coefficient is 0.996. Based on these results, we can infer that every plant species has its own regression equation which represents the linear relation between chlorophyll content and the ratio $F_{685}/F_{735}$. Therefore, as long as get the fluorescence intensity ratio $F_{685}/F_{735}$ from the fluorescence emission spectrums of the green plant leaves, we can make a fast and non-contact measurement on the chlorophyll content according to the regression equation of the certain plant. Furthermore, we also can make a fast judgment on the growing state and the activity of photosynthesis of the unknown-state leaf.

3.3. Monitor the stress effects in plants by the fluorescence intensity ratio $F_{685}/F_{735}$

Short-term or long-term stress to plants will either decrease the content of chlorophyll a in green leaves by partial chlorophyll breakdown or decrease the rate of chlorophyll accumulation. The both will result in the variation of the fluorescence intensity ratio $F_{685}/F_{735}$. So we can monitor the stress effects in plants by the ratio $F_{685}/F_{735}$. As an application to this theory, we use the method mentioned in this paper to get the fluorescence emission spectrums at the maximum fluorescence of the liana that was exposed to water stress. The details are: After watering the liana, we immediately choose one leaf of the liana to get its fluorescence emission spectrum with the foregoing experimental equipments. Then we keep the samples in dark and dry place, as well as keep the temperature remains constant, after the intervals of one and a half hours, we do the same experiment and get another two fluorescence emission spectrums of the same leaf, they are shown in Figure 6:

We can see from figure 6 that the fluorescence intensity ratio $F_{685}/F_{735}$ increased from 0.923(1) via 0.994(2) to 1.023(3) with the deeper water stress, due to a temporary loss of chlorophyll. Although the tiny variety of the ratio (as the time of water deficit is so short that the less photosynthetic activity remains working on and the limited technical condition, such as the conservation condition, the precision of instrument, etc.) induces certain relative error, the ratio $F_{685}/F_{735}$ will be a practical indicator to monitor the stress effects on plants with the improvement of related technology. By measuring the changes of the ratio $F_{685}/F_{735}$, we can also monitor the state of health or the photosynthetic activity of certain plant faster and more visually.

4. Conclusion

In this paper we first use a red light laser diode to stimulate fluorescence, further develop a new method to make a fast and non-contact measurement on the chlorophyll a content both in water and green leaves. The experimental results indicate that it is completely feasible. Compared with the traditional method, this method has some notable features and innovations: using the laser diode as an excited light not only can avoid background noise in the fluorescence emission spectrum that induces by any other excited lights, but also can be liable to miniaturize the system. It also brings higher sensitivity and accuracy by using the chlorophyll a fluorescence intensity 685 nm and the fluorescence intensity ratio $F_{685}/F_{735}$ to measure chlorophyll a content in the water and green leaves separately.
Moreover, it verified by the applications that the fluorescence intensity ratio F685/F735 is a very suitable parameter to monitor the growing state or the photosynthetic activity of green plants. It further gives a technical foundation of on-spot monitoring the chlorophyll a content in the ocean or in green leaves.

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Figure 1-1. The schematic diagram of the equipment on measuring the concentration of chlorophyll a in the solution.

Figure 1-2. The schematic diagram of the equipment on evaluating the chlorophyll content in green leaves.
Figure 2. Fluorescence emission spectrums of chlorophyll $\alpha$ solution with different concentration ($1-c_0; 2-5c_0/6; 3-5c_0/8; 4-c_0/2$), which are given for the state $c$ maximum fluorescence.

Figure 3. The relation between relative fluorescence intensity and the normalized concentration of chlorophyll $\alpha$ solution (solid line), the broken line given as a regression curve.
Figure 4. With the increasing chlorophyll content ($C_1$=1.31, $C_2$=1.63, $C_3$=1.96, $C_4$=2.42) of the bamboo, the corresponding fluorescence intensity ratio $F_{685}/F_{735}$ decreased from 1.62($R_1$) via 1.42($R_2$) to 1.02($R_4$), the broken line given as a regression curve.

Figure 5. With the increasing chlorophyll content ($C_1$=1.22, $C_2$=1.71, $C_3$=1.94, $C_4$=2.22) of the tea, the corresponding fluorescence intensity ratio $F_{685}/F_{735}$ decreased from 1.75($R_1$) via 1.45($R_2$) to 1.04($R_4$), the broken line given as a regression curve.
Figure 6. Changes in the fluorescence emission spectrum of the same leaf of the liana during different stages of water stress: 1-at the beginning of watering; 2-one and a half hour later after water deficit; 3-three hours later after water deficit.