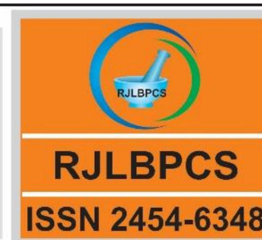


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# DEVELOPMENT OF LASER-INDUCED MULTISPECTRAL FLUORESCENCE IMAGING SYSTEM FOR STUDYING CORAL BLEACHING

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**ABSTRACT:** Coral fluorescence is an important parameter that reveals information on coral health. The current methods of fluorescence measurement require diving and proximity to the reef which may damage the reef system. Here we present a Laser-induced Multispectral Fluorescence imaging system that is capable of detecting stress and bleaching in corals by recording changes in the fluorescence emission characteristics of corals. The system uses a 457 nm diode pumped solid state (DPSS) laser for the excitation of fluorescence and an intensified charge coupled device (ICCD) camera to capture the fluorescence images at selected wavelengths using a liquid crystal tunable filter (LCTF). A pilot study carried out under controlled conditions have shown that the changes in fluorescence emission characteristics and fluorescence image intensity ratios could serve as a non-destructive tool to understand coral health without disturbing the natural habitat. The declining coral health can be detected before the appearance of visible signs of bleaching. The advantage of this system is that fluorescence information can be obtained across the entire coral surface area in the presence of ambient light in near real-time.

**KEYWORDS:** Coral bleaching, multispectral imaging, coral fluorescence, temperature stress, coral health.

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## 1. INTRODUCTION

Coral reefs are one of the most diverse ecosystems in the world, and are often referred to as "rain forest of the oceans because of their biodiversity richness and high level of primary productivity. It is estimated that reefs provide economic goods and ecosystem services valued at 400 billion a year [1]. In spite of their great biological, ecological and societal significance, coral reefs worldwide are declining as a result of both natural and anthropogenic factors [2], [3]. Coral are also known to be the most sensitive to long term climate change. Elevated temperature is one of the major causative agent of catastrophic bleaching events that is brought about by the disruption of coral – algal symbiosis [4], [5], [6], [7], [8]. Most of the mass bleaching events occur especially when elevated temperature coincide with high light intensity [9], [10]. Coral fluorescence is considered as an excellent tool to assess coral health [5], [11], [6], 12], [13]. The fluorescence studies on corals have increased considerably during the past several years. Coral fluorescence results from host fluorescent proteins as well as from the chlorophyll pigments of Zooxanthellae. Coral fluorescence studies are aimed at understanding the effect of stress elements on these fluorescent components, which can serve as indicators of coral health. Different methods are used to study coral fluorescence. Pulse Amplitude Modulated (PAM) fluorometry with short light pluse of 3μs for excitation of chlorophyll fluorescence is one of the common methods used [12], [13]. This allows for determination of effective quantum yield in the presence of sunlight. The light sources available include measuring light, actinic light, saturation pulse and far-red light. The advantage of these systems is that they are nonintrusive [14], [15]. However, as PAM fluorometers are meant to study chlorophyll fluorescence, they do not provide information on coral host pigment fluorescence. Mazel [11] measured the fluorescence emission spectra of corals with a microspectrofluorometer built around a Zeiss Standard Epifluorescence microscope. The excitation source was a xenon arc lamp and the emitted light intensity was collected over the wavelength range from 450-700 nm. Salih et al [16] obtained in vivo excitation and emission spectra of major fluorescent proteins using a fluorescence spectrophotometer with a fiber-optic probe attachment. All these methods have the advantage of enabling spectral characterization of corals without the need for protein or chlorophyll extraction. However, their inherent disadvantage was that they provide fluorescence information of a smaller area at a time, which can be time consuming when large surface areas of corals are to be screened. This problem can be overcome by using a charge coupled device (CCD) camera for fluorescence imaging. Multispectral imaging involves recording of monochrome fluorescence images at specific wavelengths of interest using discrete interference filters. But, in extreme harsh environments the use of discrete interference filters with dielectric coatings becomes risky due to presence of high humidity and salinity in the atmosphere. With the advent of tunable filters this difficulty has been overcome and multispectral imaging at narrow bands became a reality. Zawada and Jaffe [6] used a Low-light-level

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Underwater Multispectral Imaging System (LUMIS) to study the changes in fluorescence of Caribbean coral *Montastraea faveolata* during heat induced bleaching. The system combines a CCD camera with an optical splitter which allows simultaneous imaging over four discrete spectral bands. A compact cylindrical device, MultiSpec Imager$^{TM}$ transforms the image from a single collection lens into four identical copies and projects each one into a different quadrant of a single focal plane of the CCD. Coral fluorescence was induced by 4-ms excitation flash from a xenon strobe equipped with a UV-block flash tube. However, to preclude the fluorescent signals being compromised by ambient light, images were collected inside a darkroom. The requirement of dark room prevents the use of LUMIS under field conditions or for in situ imaging. In the present scenario where degradation of coral reefs due to global warming is progressing at a faster than previously thought, effective monitoring of coral reefs over a large area and for longer time period is indispensible to understand the effect of global climate change on coral reefs. Moreover, coral bleaching in the field is usually assessed by the paling of coral tissues due to bleaching [17]. Through direct observation, the extent of bleaching can be well understood but this happens after the bleaching process has already been instigated. It would be an advantage if declining coral health can be understood before the appearance of visible signs of bleaching. Though satellite remote sensing provides information on large coral cover, [18] data obtained may be vague due to inadequate resolution of satellite imagery, cloudy sky and optical properties of sea water. Hence, there is a greater need to develop an efficient, non intrusive monitoring system that can detect decline in coral health at an earlier stage, even before the appearance of visible signs of bleaching.

Here, we present such a Laser-induced Multispectral Fluorescence Imaging System (LIMFIS), capable of optical remote sensing of corals. LIMFIS utilizes a diode pumped solid- state (DPSS) laser emitting at 457nm for excitation of fluorescence and an intensified charged coupled device (ICCD) camera to record the fluorescence images of corals. A tunable filter incorporated in the LIMFIS facilitates the recording of fluorescence images at selected wavelengths from 400 to 720 nm. This camera is triggered and gated to filter out ambient light signals from the fluorescence signals thereby improving the signal-to-noise ratio (SNR). The fast gating option of ICCD and use of pulsed laser source enables data acquisition during any time of the day irrespective of light and dark hours. We have successfully evaluated the potential of LIMFIS as a tool to remotely assess pigmentation and bleaching in corals grown in a marine aquarium under laboratory conditions and the results are presented.

## 2. MATERIALS AND METHODS

### A. Prototype overview

The schematic of the LIMFIS system is shown in Fig.1. DPSS laser emitting at the sea water transmission window of 457 nm, with a CW power of 200 mW (Shanghai Dream Lasers Technology Co. Ltd., China, Model: SDL-457-200T) is used for excitation of coral fluorescence.

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Laser output is split into two beams with a beam splitter, with the main beam directed to a beam expander (Thor Labs, USA, Model: BE10M) to enlarge the beam size to two inches in diameter whereas the low power beam is directed through a glass diffuser on to a silicon photodiode connected to an oscilloscope. Fluorescence emission from corals is detected using an Intensified Charge Coupled Device (ICCD) camera, which is triggered by the laser pulse and gated to avoid interference from ambient light. The intensified CCD camera (Andor UK, Model: DH 734) consist of 1024X1024 pixels with a CCD area of 13.3X13.3 and 13μm pixel size. After the emitted light is detected and amplified, the analogue signal from the monochrome ICCD is converted to a digital signal. The process of digitization turns a measured, continuous analog signal into discrete

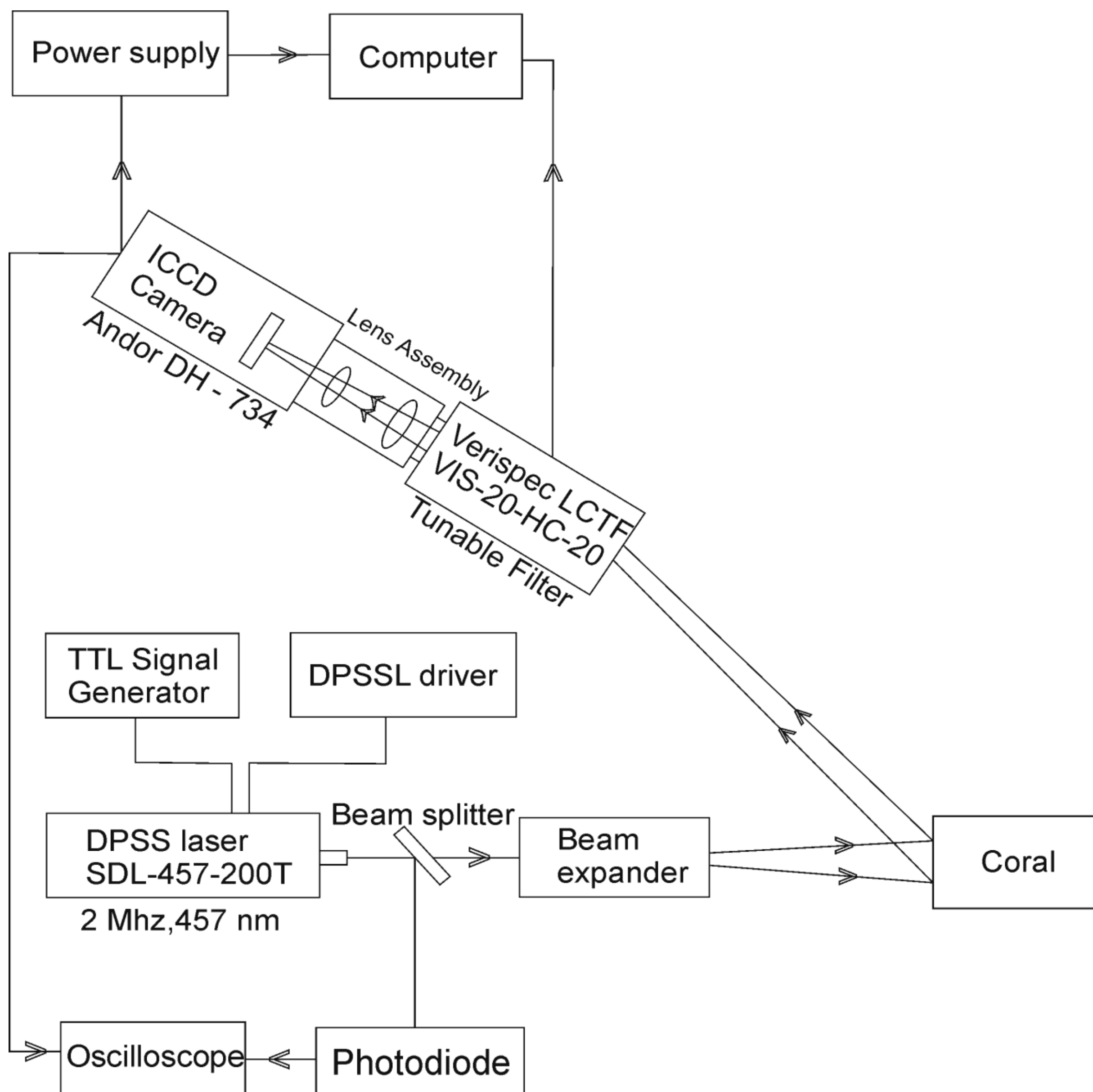


**Fig.1. Schematic of Laser Induced Multispectral Fluorescence Imaging System**

numbers based on the intensity of each pixel. This is performed using the PC interface of the camera, a PCI controller card that converts the analog signals to the digital format. Under software

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control (ANDOR SOLIS) this data is transferred to the computer memory. An external power supply unit (PS 150) that controls the current supply to the cooler to attain lowest temperature performance, is used enhances the system performance.

**B. Triggering and grating the ICCD camera**

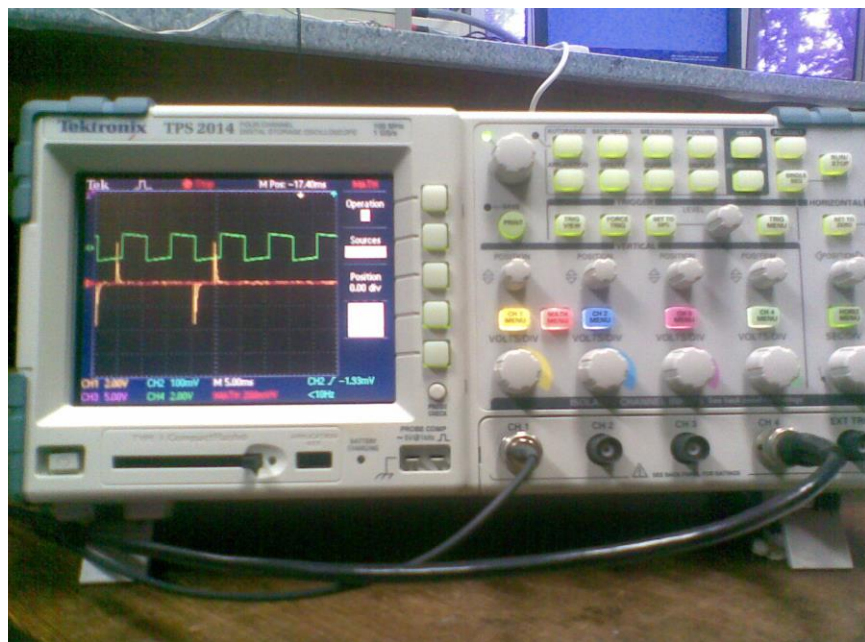


**Fig.2. The laser pulse width synchronised with the gate pulse width displayed in the CRO**

An important feature of ICCD is its fast gating options which immensely help to avoid interference from ambient light. Because of this image acquisition can be carried out at any time of the day and a dark room environment is not required. The ICCD is externally triggered by the DPSS laser. The laser is operated in the pulsed mode using TTL trigger input of 5V from the function generator at a repetition rate of 2000 Hz. A photo diode assembly along with a digital oscilloscope determines the laser pulse width and synchronizes it with the camera gate timings, which is shown in Fig. 2. The laser pulse width and camera gate timings were synchronized by adjusting the delay. The ICCD camera gate was ON during the Laser ON period. TTL width of 500 ms with a gate pulse delay of 250 us and MCP gain 96 was found to be suited for ex situ imaging. In order to capture the fluorescence images at various emission wavelengths, the MFIS is incorporated with a liquid crystal tunable filter (LCTF) that can be tuned to any wavelength between 400 to 720 nm (Cambridge Research & Instrumentation Inc, USA; Varispec LCTF Model VIS-20-20). The bandwidth of the LCTF is 20 nm. The filters function like high quality interference filters, but the wavelength of the transmitted light transmitted are electronically tunable and allow for the rapid, vibration less selection of any wavelength in the visible (VIS) or near infrared (NIR) region. The LCTF employs electronically controlled liquid crystal elements to select a transmitted wavelength range while blocking all others. Filter transmittance is sensitive to polarization of the input beam, and is increased by a factor of two if the input beam is polarized
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along the axis of the input polarizer. The LIMFIS has two retractable mirror mounts, with provision for multi-axial tilt; one for guiding the laser beam to the target and another for collection of coral fluorescence. A trolley has been built to house the entire system, with the upper deck occupied by the LCD monitor and the Tektronix oscilloscope, the top cabinet by the ICCD camera and LCTF, the middle cabinet by the laser, photodiode circuit and the beam expander and the bottom cabinet by the computer, trigger generator and the ICCD thermoelectric cooling system (Fig.4.). The LIMFIS is connected to an inverter power supply, with two 12 VDC batteries that can support continuous operation of the LIMFIS under field conditions for 4 hours without recharge. Image collection and analysis is done by using Andor-Solis software. The experimental set up needs to be kept undisturbed during the LIF image acquisition of an organism sequentially at different emission peaks and the fluorescence image intensity ratios are computed. Afterwards a region of interest (ROI) in the imaged area is taken to calculate real-time quantities like mean and standard deviation of the image ratio.

## 3. RESULTS AND DISCUSSION

### Assessment of LIMFIS

LIF imaging of corals was done different species of corals collected and maintained in a marine aquarium at the Central Marine Fisheries Research Institute (CMFRI), Vizhinjam, Trivandrum. The corals were grown in a large tank under controlled conditions with adequate lighting and aeration. Fluorescence imaging was conducted on hard corals since they are more significant as reef formers. Studies were conducted on partially bleached samples of *Favia matthaii* and *Goniastrea retiformis* are shown in fig. 3. A mirror deflects the expanded laser beam on to the coral while another mirror guides the fluorescence emission from the coral to the ICCD camera through the LCTF, fitted on the top deck of the system. The camera captures the fluorescence image from another mirror mounted on the LIMFIS. The experimental set up is shown in fig.4.

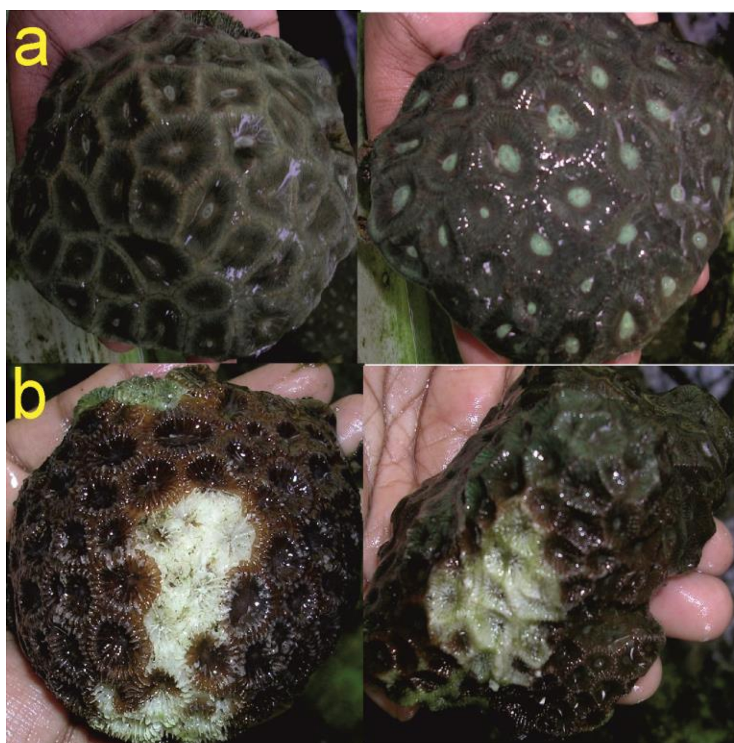


**Fig.3.Healthy and bleached samples of *Favia mathaii and Goniastrea retiformis***

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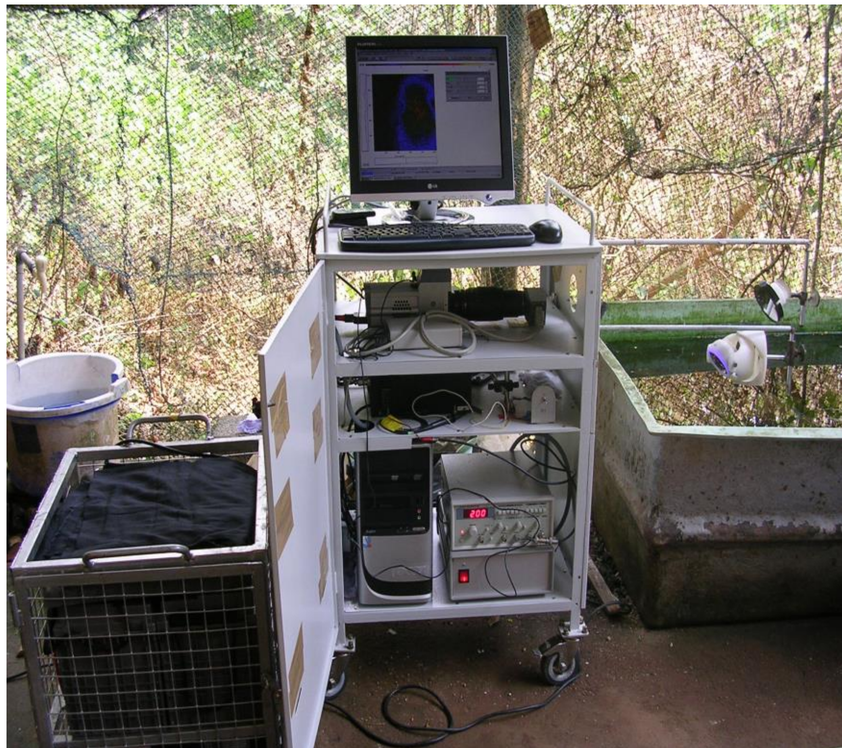

**Fig.4. Experimental setup of LIF imaging of corals using LIMFIS at CMFRI, Vizhinjam**

The fluorescence emission of these corals consists of a host pigment emission at 510 nm and chlorophyll emissions at 680 and 720 nm. Hence, the fluorescence images of healthy and bleached samples were taken at 510, 680 and 720 nm. The 510 nm emission was the most intense followed by 680 and 720nm. Moreover, the intensity of the host pigment emission at 510 nm was highest at the mouth region which is shown in fig.5. The F 510/720, F 510/680, and F680/720 ratio images of partially bleached corals were obtained from images captured. Among the three above mentioned fluorescence intensity ratios, F680/F720 varied the most with bleaching. Fig. 6 shows the monochrome and false coloured images recorded at 680 and 720 nm along with the F680/F720 image. The monochrome images obtained were false coloured for better visualisation. Though only a small area appears affected or bleached to the naked eye the

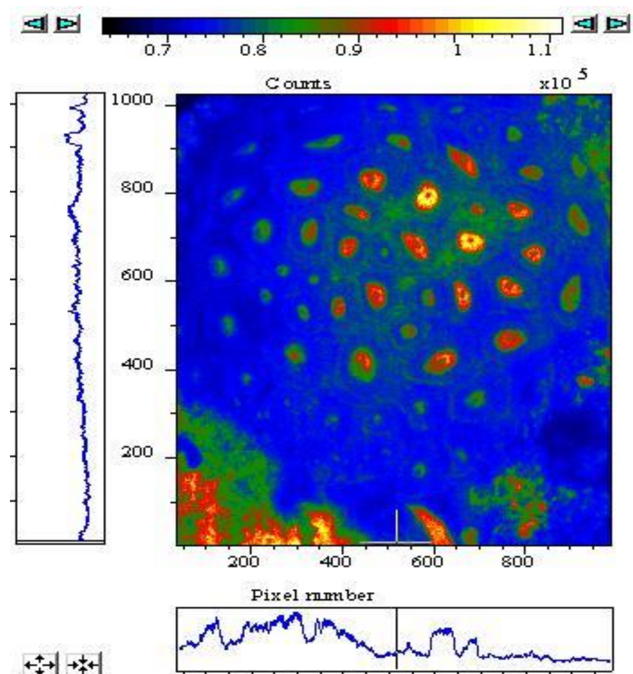


**Fig.5. LIF image at 510 nm of a healthy Favia species. The 510 nm host pigment emission is highest at the mouth region.**

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F680/F720 image clearly illustrate that a much larger area is affected. The visibly bleached region appears to be blue as this region showed minimum fluorescence intensity and the surrounding affected region appears to be green in the F680/F720 image.

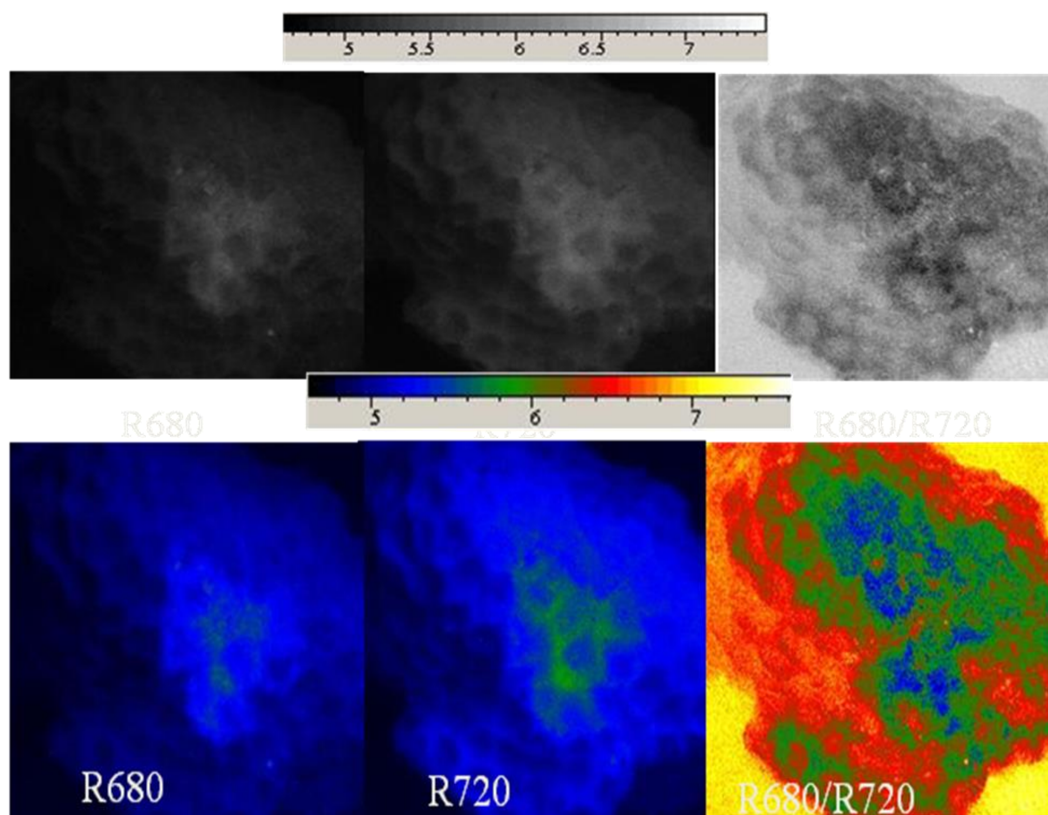


**Fig.6. Monochrome and false coloured images recorded at 680 and 720 nm along with the F680/F720 ratio image**

The regions of interest were taken from each ratio image of partially bleached corals. From the region of interest the mean ratio values were determined. In order to understand the variance of fluorescence signatures with the progress of bleaching ratio images were computed from healthy (H), bleached (B) and its adjoining (A) region. The fluorescence ratio of 680 and 720 nm intensity (F680/F720) determined from the ratio image showed maximum variation with bleaching. Bleaching results from the expulsion of symbiotic dinoflagellate, Zooxanthellae due to stress. The two peaks around 685 and 740 nm are attributed to the chlorophyll pigments associated with Photosystem (PS) II and PS I, respectively. Hence, the changes in fluorescence with bleaching are more prominent at 680 and 720 nm. In the F680/720 fluorescence intensity ratios calculated, *Goniastrea retiformis* showed a variance of 4.12% between H and A and a variance of 2.06% between H and B, which is plotted in fig.7. *Favia matthaii* showed a variance of 6.12% between H and A and a variance of 10.2% between H and B. This variation in the F680/720 ratio between healthy region, region adjacent to bleached region and bleached region opens up the possibility of detecting coral bleaching at an early stage.

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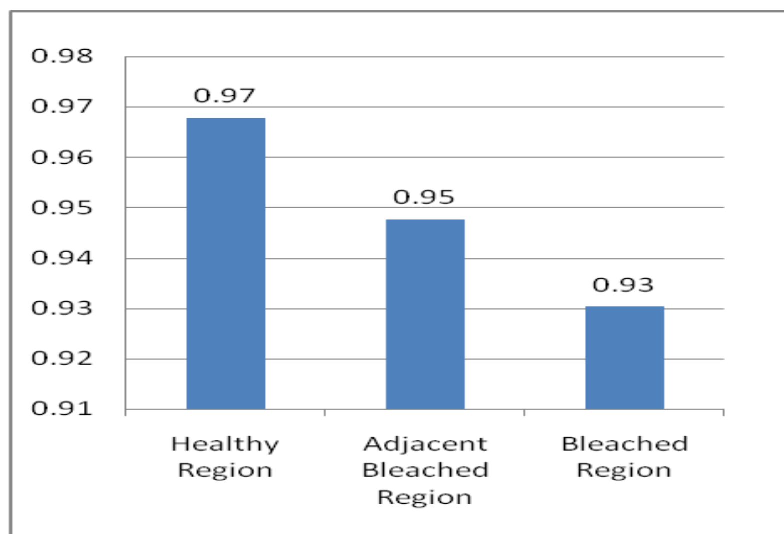


**Fig.7. Chart showing the variance in F680/F720 ratio during bleaching progression**

## DISCUSSION

In the present scenario, coral reef ecosystems are under severe threat from both natural and anthropogenic activities. As corals are extremely sensitive to long term climate change, rise in temperature and ocean acidification are taking its toll on coral reefs. Any stress on corals leads to coral bleaching. As the visible effects of bleaching appear only after severe damage to the tissues, it is necessary to detect the bleaching effect at the early stage, which would require the capability to detect stress before the appearance of visible symptoms. The present study has shown that fluorescence data from LIMFIS can be used to detect coral bleaching at an earlier stage. The data gathered would also help to understand the distribution of fluorescent pigments in corals. The fluorescence emission at 510 nm was found to be the most intense at the polyp centers and weakened towards the outside. Moreover, the fluorescence intensity was almost nil in the area between the polyps. The chlorophyll fluorescence however, was found to be distributed uniformly over the coral surface. In an earlier study, Salih [19] concluded that the fluorescent intensity of fluorescent pigments are greater at the polyp centers compared to the corallite rims. Similarly, Zawada and Jaffe [6] has reported that the 515 and 575 nm signals from the fluorescent proteins emanated from the polyp centers and the fluorescence intensity rapidly decreased in the area between the corals. Chlorophyll fluorescence on the other hand was evenly distributed across the coral surface, diminishing at the polyp centers, where the 515 and 575 nm emissions are the most powerful. Moreover, most of the corals exhibited uniform chlorophyll fluorescence across the entire coral surface. One of the important observations is that chlorophyll fluorescence decreases with stress and bleaching. This might be due to the reduction in photosynthetic pigments associated with bleaching. Similarly, Lee [20] reported that the effect of temperature elevation was more pronounced in chlorophyll (red) fluorescence, with fairly rapid declines following the onset

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of temperature increase. Within 6 h, chlorophyll fluorescence in all elevated temperature regimes was significantly lower than the initial intensities. At 38°C, the rate of decline was most distinct, remaining at about 35% between time steps over the duration of the experiment. Moreover, the Chlorophyll *a* fluorescence intensity in cells exposed to this temperature was significantly lower than that of the other treatments. In the dinoflagellates, each quantum of light absorbed by a chlorophyll molecule rises an electron from the ground state to an excited state. Upon deactivation from a chlorophyll a molecule from the excited state 1 to ground state, a small proportion of the excitation energy is dissipated as red fluorescence. Zooxanthellae contain auto fluorescent photosynthetic pigments (chlorophyll a and c), which produces red fluorescence signal at 680 nm and 720 nm after excitation with a blue light source [21]. The indicator function of chlorophyll fluorescence arises from the fact that fluorescence emission is complementary to alternative path ways of de-excitation, which are primarily photochemistry and heat dissipation. Compared to healthy corals, bleached corals were known to contain greatly reduced quantities of photosynthetic pigments, chlorophyll c, peridinin and diadinoxanthin [22]. Hardy et al [5] observed similar reduction in the chlorophyll fluorescence in the coral species subjected *Solenastrea bournoni* when subjected to temperature stress. They also quantified the total photosynthetic pigments in healthy and bleached samples and found that the concentration of photosynthetic pigments declined in corals subjected to stress. This reduction in the pigments may be due to the reduction in chlorophyll pigments or due to the expulsion of Zooxanthellae. The system can be used for active remote sensing of coral reefs. However, under field condition water movement was found to hinder clear image acquisition. This can be overcome by using imaging fibers. The fiber guides the laser beam for excitation of fluorescence to the corals and another fiber transmit the fluorescence image through the fiber to the camera. This way the water movement does not interfere with image acquisition. The fast gating option of ICCD and use of pulsed laser source enables data acquisition during any time of the day irrespective of light and dark hours. This prevents fluorescent signals being compromised by ambient light. Once the camera gate timings and laser pulses are synchronised, the camera gate will be ON during the Laser ON period minimising the interference from ambient light. This is especially applicable for imaging under field conditions which will be flooded by sunlight.

## 4. CONCLUSION

Corals throughout the world are suffering from an onslaught of both natural and human disturbances that in concert over the last few decades have degraded or destroyed many reefs. They are also believed to be the most sensitive ecosystem to long term climate change. Thus it is necessary to detect the bleaching effect in the early stage, which would require the capability to detect stress before the appearance of visible symptoms so that one could alleviate the causes of stress before irreversible damage occurs to corals. From the study it is evident that fluorescence

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data from LIMFIS can be used to detect coral bleaching at an earlier stage. Moreover, the data gathered would also help to understand the distribution of fluorescent pigments in corals. The developed system (LIMFIS) can also be used for active fluorescence imaging of vegetation and other targets including a whole range of reef organisms, algae and other bottom dwellers. The LIMFIS has an added advantage of being completely non-intrusive, since the system can be operated away from the target.

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