Specific fluorescence and intrinsic lasing within microalgae to monitor biofuel production (LASinAFuel)

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ABSTRACT
Several microalgae species, able to accumulate large amount of oils, can be exploited as valuable alternative source for renewable fuel, chemicals and food. Yet, a rapid and efficient diagnostic tool to monitor oil accumulation in microalgae is needed to maximize productivity and boost competitiveness of this renewable source. Here we show how extrinsic fluorochromes can be used to monitor properties of small samples of microalgae cultures, with preliminary and promising observations of lasing. Among many dyes, we identify the best performing dye – lab-prepared, fully novel in the field – which is being patented to protect future developments of this research.

Keywords: Microalgae Chlorella Vulgaris; Fluorescence and Dye-Lasing; Renewable Fuels and Chemicals.

1. INTRODUCTION

- Microalgae biomass has a strong potential as alternative, 3rd generation non crop-based biofuel. Biofuel production from microalgal biomass directly tackles the paramount problem of climate change: fuel is produced directly from carbon fixation, drastically cutting the CO_2 footprint of energy production. *Algae-based biomass is promising also for renewable production of chemicals, food, feed and materials*. Yet, a major limitation for scale-up to large-area cultivation, setting a technological barrier for current or potential markets, *is the availability of systems for efficient monitoring of the process at industrial scale*. While microalgae biomass cultivated at the lab-scale reaches high and reproducible yields, monitoring and control of large-scale cultivation systems is complicated, especially for complex factor as lipids content, negatively affecting productivity and thus competitiveness.
- The breakthrough goal of LASinAFuel is to realize *intrinsic biolaser sensors in unicellular microalgae based on WGM microresonators*, to develop a fast analysis of the status of these organisms. Their oil droplets are perfect candidates for realization of intracellular WGM microlasers, with great potential for fast and accurate sensing of their size, shape and surface interactions. A wealth of information may then be extracted related to microalgal lifecycle and growth, oil accumulation, mechanical forces and physical stresses occurring on the individual oil droplet. In addition, dyes distributing differently in the various algal microenvironments will be exploited to develop *an easy, cheap and user-friendly method for estimation of oil amount* per microalgal unit. The method will be based on a colorimetric test to perform with a smartphone app. Being ratiometric or using absolute photophysical properties such as fluorescence lifetime or anisotropy, such a method will not need calibration since the signal directly provides the oil content of the sample.
- Ultimately, the envisioned potential of LASinAFuel is the development of a fast and sensitive detection tool to apply in all *high-throughput screenings of oil-producing microalgae*, both for research and for industrial large-scale cultivations. To this end, we have (i) prepared and characterized suitable dye candidates, (ii) developed an internalization method that leads to high dye-doping of microalgae and selected the most promising dyes for efficient dye-doping, (iii) developed a fast and ratiometric measure to evaluate the average oil content and (iv) obtained preliminary laser emission signal from microalgae samples.

2. STATE OF THE ART

EU's renewable energy target for transportation sector (RED II directive 2021-2030) sets a target of 14% of fuels to be renewable by 2030, with a binding 3.5% sub-target

on non-crop based "advanced" biofuels. Among them biofuels produced from *algae biomass have a strong potential as alternative, non-crop based biofuel*, as recognized in the Bioenergy Materials Roadmap within the SET (Strategic Energy Technology) Plan.
Microalgae are a largely diverged group of unicellular photosynthetic organisms with some species showing a strong ability to accumulate lipids, a suitable feedstock for production of biofuels. *Lipid accumulation depends on several environmental parameters* such as light intensity, nutrients and CO_2 availability.[1] A simple method to monitor oil accumulation on large-scale is presently not available, limiting control of large-scale systems, ultimately affecting productivity.
Lasing from cells in the presence of various types of resonators has recently been subject of thorough investigation, with cells playing a role as either passive dye containers or active components for light confinement.[2] In our lab, we recently observed that highly stained biological cells can confine light and produce laser action in absence of any conventional optical cavity.
Yet, at present, no laser emission has been observed from dye doped microalgae, and no relation with oil accumulation has been hypothesized.
A special class of micro-resonators are spheres with diameter ranging from few to hundred microns in which light can be trapped via total internal reflection, originating resonant standing waves (whispering-gallery modes, WGMs). An important aspect of WGM microcavities is that resonant frequencies respond in real time: *resonating frequencies are tuned with the optical path of the light*, when either the refractive index or the *physical size and shape of the resonator change*. As such, WGMs exhibit unique properties is the area of sensing of small forces or for detection of surface interactions.[3]

3. BREAKTHROUGH CHARACTER OF THE PROJECT

In this project, we aim to realize intrinsic biolasers sensors in unicellular microalgae based on WGM microresonators, to develop a fast analysis of the status of these organisms. Their *oil droplets are perfect candidates for realization of intracellular WGM microlasers*, with great potential for fast and accurate sensing of their size, shape and surface interactions. A wealth of information can then be extracted related to microalgal lifecycle and growth, oil accumulation level, mechanical forces and physical stresses occurring on the individual oil droplet.
This pioneering research can then readily be translated in application: biolasers in microalgal oil droplets can be a revolutionary new *tool to monitor oil accumulation and thus increasing control on biofuel production process*, with resolution down to the single cell. This breakthrough tool is conceived first for specialized laboratories, but with specific protocols and guidelines it will be translatable to non-specialized use, to optimize cultivation and growth conditions of algae at large scale, with the aim to synchronize their biofuel accumulation and increase the energy yield of the bioreactor.
In fact, knowledge of the distribution of different dyes in the various environments of the algae will allow us to develop an easy and user-friendly method for estimation of oil amount per microalgal unit. The method will be based on a colorimetric (ratiometric emission) test that can be performed with an app for smartphone, without the need for an intensity calibration curve. The *average colour emitted or reflected by the sample will be decoded* by the smartphone under either ultraviolet (fluorescence-based test) or visible light (chromatographic test).

4. PROJECT RESULTS

We have used two types of microalgae, i.e. *Chlorella vulgaris* and *Nannochloropsis gaditana*, largely employed for oil accumulation. The small cellular size of *Nannochloropsis* (1-3 micron diameter) complicates the characterization of the localization of the dyes inside the microalgae with confocal microscopy. Therefore, we focused our studies on *Chlorella vulgaris* microalgae, a well characterized strain with diameter in the range 3-6 microns. Adapting the developed strategies to other algal strains is expected to be straightforward and will be part of future research.
A list of dyes, either commercial or synthesised in our laboratories, has been identified, based on both their chemical and photophysical properties, with the aim to heavily dope microalgae with fluorescent stains that, upon pulsed excitation (8 ns, 532 nm) could undergo laser action.
The fluorescent dyes, suitable candidates for lasing, are listed in table 1. Among them, a few have demonstrated superior internalization capabilities, based on analysis with confocal microscope.

Tab. 1. List of synthetic and commercial fluorescent dyes tested for internalization in Chlorella Vulgaris. Internalization rate is given in range 0-3 where 0 = scarce, 1 = moderate, 2 = good, 3= excellent.

Dye	Internalization (0-3)	Acronym	Type
Rhodamine 6G	3	R6G	commercial
RhodamineB	1	RB	commercial
Rhodamine101	3	R101	commercial
Nile Red	2	NR	commercial
Neutral Red	1	NeR	commercial
Perylene Jeffamine	3	PJ	Synthetic
RB-Oleyl	3	RO	Synthetic
RB-Hexyl	2	RH	Synthetic

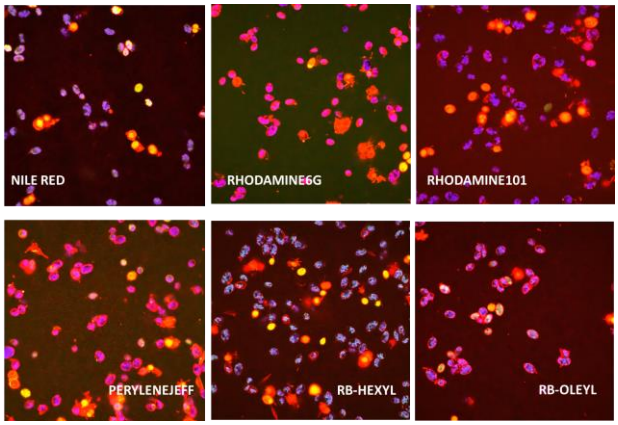

Fig. 1. Confocal micrographs taken with three acquisition channels (green: λ_{exc} = 489 nm, λ_{em} = 525/50 nm; red: λ_{exc} = 562 nm, λ_{em} = 595/50 nm; violet: λ_{exc} = 638 nm, λ_{em} = 700/75 nm) and constant acquisition parameters of N-deprivated Chlorella samples. R6G and PJ show the largest internalization (colocalization of red and violet). RO and PJ show the largest accumulation in cell membranes (red cell contour)At present, preliminary observations of lasing sharp peaks have been performed with R6G and R101, even if the sensitivity of the measurement is at the multi-cell level and needs to be improved to reach the individual cell level.

The doping method is simple and scalable to larger quantities, and could also be miniaturized for small or micro- fluidic devices: a given amount of algal culture is mixed 1:0.5 with a concentrated solution of the dye (range 1-5 mM) in an organic solvent of choice among methanol, DMSO and acetone. After a short incubation of ca. 5 minutes the dye-doped algae can be separated from dye eventually dispersed in water via 3 centrifugation cycles (5000 rpm, 10 minutes). The obtained samples of dye-doped microalgae are then ready for further analysis and testing.

The stained algae have been characterized by means of spectrophotometry, spectrofluorimetry and fluorescence imaging methods.

Confocal microscopy of N- *Chlorella* cells allowed us to quantitatively evaluate the amount of dyes internalized and their localization within the cells. Undoped algal strains have also been characterized as blank references. As shown in Fig. 1, the dyes R6G and PJ are the best stains for the internal part of the cells, and likely more specifically for the oil droplets. The dyes RO and PJ are very good stains for the outer membranes of the algal cells. Among them, as summarized in table 1, the best candidates for microalgae doping and lasing are R6G, R101, PJ and RO.

Fluorometric studies have revealed promising aspects of the developed staining procedure and of the selected dyes. We have comparatively studied *Chlorella* cells grown under nitrogen deprivation ("N-" samples, a condition that stimulates strong accumulation of oil, while also depleting the synthesis of chlorophylls) and Chlorella grown in presence of N sources, with normal (low) accumulation of oil. The goal of this work package was to find a dye featuring the largest contrast between algae accumulating excess of oil ("N-" samples) and algae with normal oil vesicles. As expected, a marked decrease of chlorophyll absorbance and fluorescence was observed in N (nitrogen)-stressed *Chlorella*.

By using a dye whose fluorescence is enhanced by oil accumulation, a ratiometric sensor can be developed. Among the tested dyes, PJ has the most striking capability to provide a strong and efficient ratiometric signal, allowing quantitative assessment of oil accumulation in N-stressed Chlorella microalgae (Fig. 2). In addition, the two emission signals from PJ and from chlorophylls are well separated and therefore suitable for RGB decoding with smartphone cameras.

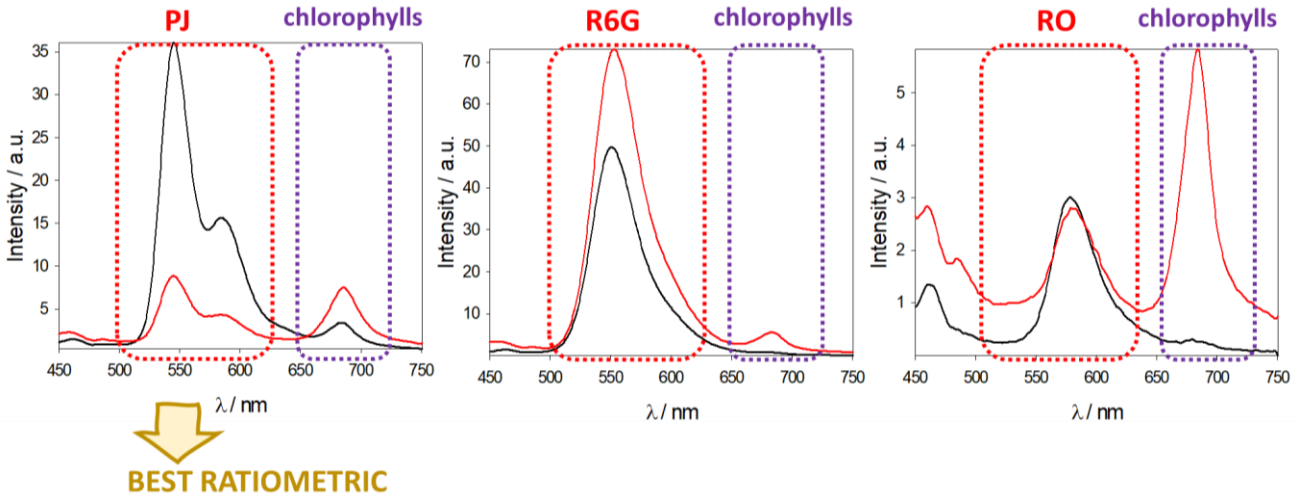

Fig. 2. Fluorescence spectra (λ_{exc}= 400 nm) of *Chlorella* samples under normal growth conditions (red lines) and N-deprivated growth conditions (black lines) stained with three dyes featuring ratiometric responses: PJ (left), R6G (center) and RO (right). PJ shows the largest ratiometric response (low PJ fluorescence and high chlorophyll fluorescence in reference *Chlorella*, high PJ fluorescence and low chlorophyll fluorescence in N- *Chlorella*). The two emissions are well separated and therefore suitable for RGB decoding with smartphone cameras.

Beside ratiometric response, absolute photophysical parameters exist that can provide direct information without need for calibration procedures. We have found that two absolute parameters of the tested dyes, i.e. fluorescence lifetime (τ) and fluorescence anisotropy (r), also show a large contrast between the non-stressed algae and the N-stressed samples (Fig. 3). Also in this case, the dye PJ shows the largest contrast between samples with different lipids accumulation (control and N-) and thus is the best candidate for sensitive monitoring of oil accumulation.
The dye PJ is a synthetic dye obtained in our laboratories and recently published;[4] the use in algal staining to obtain a ratiometric sensor for monitoring oil accumulation is completely novel and thus patentable. Patent filing is currently being assessed, also with the aim to protect future developments of these results, as detailed in section 5.

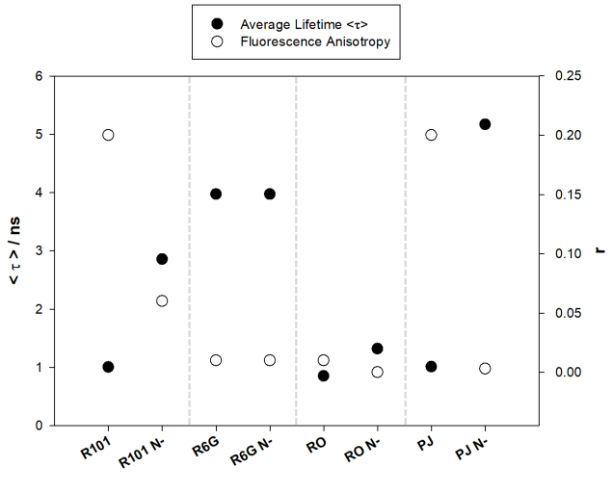

Fig. 3. Absolute photophysical parameters (fluorescence lifetime (t) and anisotropy (r)) of control and N-stressed *Chlorella* cells (N-) doped with dyes R101, R6G, RO and PJ. PJ features the largest contrast between control and N-*Chlorella* samples.

5. FUTURE PROJECT VISION

The next-future plans of LASinAFuel for ATTRACT Phase 2 are related to the implementation of the novel working principles obtained in Phase 1 into ready-to-use monitoring devices.
The monitoring devices will be based on the three different working principles as stemming from Phase 1 (ratiometric, absolute parameters and lasing) and on the use of the to-be patented synthetic dye PJ.
A relevant part of the project in Phase 2 will concern engineering and optimization of the devices, with a focus on integrating the device in miniaturized fluidic systems, paving the way to fully automatic monitoring of small and large biofuel power and chemical plants.

5.1. Technology Scaling

The present proposal has reached the proof of concept stage (TRL3). TRL scaling will proceed with use of the approach for monitoring algae cultures in lab-scale photobioreactors, in collaboration with the partner TMCI Padovan spa (TRL4). Further development to TRL5-6 will require different additional steps, and partners. Key points of this technology scaling will be: i) development of monitoring devices in collaboration with engineers and app developers, and with other ATTRACT Phase 1 project partners (see 5.2). ii), the devices will be interfaced with fluidic circuits, to obtain live, fully automatic and parallel monitoring capabilities. iii), test of devices with algae from *industrial photobioreactors, in collaboration with partner TMCI Padovan spa* (technology proven in industrially relevant environment, TRL6).

5.2. Project Synergies and Outreach

In ATTRACT Phase 2, LASinAFuel will activate a strong collaboration with a group of engineers and with an app developer to design the various devices for monitoring biofuel accumulation during algal growth. This cooperation will continue with integration of the devices in fluidic systems. These synergies will be essential for reducing the costs and make the monitoring system user friendly.
Collaborations with ATTRACT Phase 1 funded projects will synergically boost the final technology innovation: the monitoring devices need efficient photon sensors for all proposed signalling mechanisms. Phase1 projects "Real-time fluorescence lifetime acquisition system" (RfLAS) and "FLuorescence analysis speedup to extremely high rates" (FLASH) develop extremely attractive sensing devices, with the potential to boost the innovation level of LASinAFuel even higher.
Concerning dissemination, we aim to initiate testing of our technology in on-field activities across Europe, in order to (i) outreach and liaise with the community of biomass producers, boost their productivity and to (ii) disseminate our findings and the stemming technology both in the society and in the education system (schools and trainings).

5.3. Technology application and demonstration cases

In ATTRACT Phase 2 we aim to increase the TRL of our monitoring tool from TRL3 to TRL5-6, to reach the on-field test and demonstrate the easiness and feasibility of the proposed monitoring method. Quantitative evaluation of the biofuel field production with and without monitoring tool will be initiated, with final evaluation in the 2-3 years timescale.
As mentioned in the introduction, enhancing the efficiency of algal biomass can have a huge impact in the market growth of this sustainable source with strong

potential impact on both the energy sector (biofuels) and in food, feed or green chemistry (renewable source of high value oils and TAGs). As a consequence, the progresses of this research are tightly related to the following Societal Challenges identified by the EU:

- *Secure, clean and efficient energy;*
- *Climate action, environment, resource efficiency and raw materials;*

5.4. Technology commercialization

Commercialization of our technology will consist of three main sectors, i.e., (i) commercialization of the chemical reagents (patented dye), (ii) commercialization of the monitoring device and (iii) commercialization of the app.
In addition, different technologies may be proposed as the core-technology of the sensing device, based on either (i) ratiometric sensing (the simplest and cheapest technology), (ii) absolute average parameters (lifetime and/or anisotropy) or (iii) lasing (the technique with highest potential, likely also the most expensive).
As for the chemical reagents and chemosensors, we are now patenting the use of PJ, a dye recently synthetized in our laboratories, as a sensor for oil accumulation in microalgae. As detailed in the results section, PJ shows both a very good fluorescent ratiometric response, suitable for a simple tool such as a smartphone app, and absolute responses in lifetime and anisotropy, providing direct oil quantification with dedicated instrumentation.
The *commercial application of this dye will be possible independently from the monitoring device and app.* A new, improved, dye for monitoring oil accumulation in algae, even without the development of monitoring devices, would already be of high interest for a few hundreds specialized labs.

5.4. Envisioned risks

The three technology working principles are characterized by very different risk levels.

- The ratiometric approach appears already very solid, with only remaining risks in the translation of the principle onto the RGB colours of the smartphone camera.
- The absolute parameters (lifetime and anisotropy) are based on well consolidated techniques. Here the main risk stems from device engineering, in particular the time-resolved photon sensor. The synergistic collaboration with above-mentioned projects (RfLAS and FLASH) is a powerful mitigation strategy, with extra-gain owing to their intrinsic innovation potential.
- The last approach, based on lasing, is still the furthest from application, with a great deal of parameters to be adjusted to optimize magnitude of the signal and sensitivity of the technique. TRL 3-4 are still to be consolidated. Advances to TRL 5 and higher, via implementation in a fluidic or in a scaled-up device, will need dedicated solutions. Yet, similar issues have already been faced in many other applications with similar laser technologies, therefore a library of useful solutions can be found in the literature and in the market.

5.5. Liaison with Student Teams and Socio-Economic Study

As in Phase 1, we will keep collaborating with MSc. Level student teams to activate training and exchange of ideas in the field of sustainable chemicals and energy production. We currently have 5-10 MSc. Students per year performing 4-12 months research each. Besides our supervision, PhD students and postdocs also contribute supporting, supervising and guiding their learning and creative research steps.
The expertise acquired during training will enable them as possible innovators, driving new economic initiatives in the strategic field of algae biotech for the sustainable production of food, feed, additives and fuels.
Concerning the socio-economic study, we will contribute with interviews, reports, and recordings of on-field activities.

6. ACKNOWLEDGEMENT

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