Visualisation of electrochemical processes by coupled electrochemistry and fluorescence microscopy

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Abstract
The in situ coupling between electrochemistry and fluorescence microscopy is gaining popularity in the electrochemical community as a tool to image electrode processes in real time, providing useful spatial information which is not accessible by purely electrochemical methods. In recent years, new applications of electrochemical-fluorescence microscopy coupling have emerged, together with methodological developments that have expanded the possibilities of this technique. These recent trends are discussed in the present contribution, with an emphasis on the fluorogenic probes being employed.

Scope
The coupling of optical microscopies with electrochemistry offers a non-invasive way to visualise electrochemical processes in situ and in real time [1,2]. Compared to local probe microscopies, optical microscopies generally have a lower spatial resolution but a larger field of view and usually a better time resolution. A prime interest of such coupling is that in classical electrochemical methods, the measured signal is always averaged over the entire electrode surface and, except for purely surface-based phenomena, directly influenced by transport phenomena occurring in solution. The information on possible local peculiarities at the electrode surface or in solution is thus hidden or only indirectly accessible by sole electrochemistry. These techniques are well adapted to the study of systems presenting macroscopic heterogeneities and afford the monitoring of phenomena in 3D. They are gaining popularity in diverse fields of electrochemistry, notably in electrochemical energy systems [2]. Many optical microscopies can be coupled to electrochemistry, each encompassing specific optical configurations; the interested reader is referred to recent comprehensive reviews [3,4]. Herein, the focus is exclusively given on the in situ coupling between electrochemistry and fluorescence microscopy (EC-FM). Some important concepts were introduced in a previous review [5], and the present contribution discusses some of the most recent trends in the field. The first section is devoted to the type of electrochemical processes that have been investigated, and the second section to the specific fluorogenic probes that have been employed. Single-molecule and super-resolution fluorescence, which are attracting a considerable interest in the electrochemistry community, are considered beyond the limited scope of the present discussion but have been thoroughly reviewed in Ref. [6].

Electrochemical processes
Space- and/or time-resolved progress of electrochemical reactions
EC-FM is particularly adapted to provide spatial and temporal information regarding transport phenomena and the occurrence of concentration gradients associated with specific electrochemical processes. Although sole diffusion is often considered in fundamental investigations under well-defined geometries, migration and convection are also relevant, especially when higher current densities or more complex cell architectures such as porous electrodes, gas diffusion layers, membrane-electrode assemblies, etc., are considered for more applied processes.

Provided a suitable fluorogenic probe is identified (vide infra), the progress of an electrochemical reaction and its impact on the concentration profiles in the vicinity of the electrode can be monitored by fluorescence microscopy in real time and 2D or 3D. The underlying assumption is that the measured fluorescence signal (intensity, intensity ratio, lifetime, etc.) is quantitatively related to an amount concentration of substance such as the concentration of the fluorescent species or
the pH. This assumption is sufficient for semi-quantitative analyses (“the pH increases/decreases when ... ”, “the reaction front propagates in the direction of ... ”), but should be quantitatively validated through calibration curves if accurate values are sought after (“the pH value at this x, y, z position is ... ”, “the fluorophore concentration increases up to ... μM”). Such calibration should preferably be based on experiments conducted directly in the EC-FM cell rather than in a fluorescence spectrophotometer because the cell design (reflection from the electrode, tilt or drift effects, etc.) can influence the measured signal [7].

Following the seminal work of Unwin and colleagues [8,9], EC-FM in the confocal mode has been used to reconstruct in 3D the reaction layer around an electrode from fluorescence intensity maps [7,10–12] (Scheme 1a). Typical electrochemical cases comprising a direct electrochemically induced fluorescence-ON [12], a fluorescence-OFF [11], and a comproportionation reaction [11] have been documented, as well as the indirect modulation of a pH-sensitive fluorogenic probe induced by the electrochemical reduction of oxygen [7]. In this latter work, specific attention is paid to the time evolution of pH and the influence of the buffering conditions.

An advanced implementation of this 3D confocal reconstruction has been reported by Tassy et al. [10], who were able to visualise the reaction layer generated by a film of immobilised bilirubin oxidase under enzymatic catalysis conditions.

By confining the electrochemical cell geometry to two dimensions, widefield microscopy has been employed by the group of Compton [13–15] to study the spatial extent of the reaction layer under various conditions. In particular, notable differences were noticed between a direct and a mediated electrochemical reaction [13], and chemical means to confine the pH gradient were illustrated [14,15].

Macroscopic fluid motion has also been evidenced and studied by EC-FM [16–18]. Afshar Farniya et al. [17] and more recently Ashaju et al. [18] have looked at flows generated by electrocatalytic reactions, employing pH-sensitive fluorescent probes. Scida et al. [16], still using a pH indicator, studied the coupling between electrochemical and electrokinetic phenomena at bipolar electrodes.

The early stages of water electrolysis were investigated at the single-molecule level by Zhang and colleagues [19] who followed the formation of nanobubbles by EC-FM. Using very low concentrations of the classical Rhodamine 6G dye, they took advantage of the distance-dependent quenching effect of the electrode [20] and of the employed total internal reflection fluorescence setup to record fluorescence signals arising exclusively from fluorophores trapped at the gaseous nanobubble—liquid interfaces.

Two recent contributions of particular interest deal with genuinely operando visualisation of electrochemical processes [21,22]. Fuladpanjeh-Hojaghan et al. [21]...
have constructed a one compartment transparent electrochemical cell enabling the microscopic imaging by laser scanning confocal fluorescence of the spatio-temporal pH distribution in the cell (Scheme 1b). In their observations of electrocoagulation processes, they were able to assess the influences of current density, natural convection and gas evolution on the evolution of pH with time [22] monitored the flow of electrolyte within porous electrodes of a redox-flow battery under working conditions. Regions of distinct mass transport regimes were identified.

Fluorescent reporting of electrochemical activity
Aside of the spatial visualisation of electrochemical reactions, another frequent use of EC-FM since its early days [23–25] is the assessment of the electrochemical activity of a specific catalyst or a specific region of a heterogeneous electrode. In this case, the fluorescence intensity is used as a (semi-)quantitative descriptor of the electroactivity. An early example of electrocatalyst screening by EC-FM is given by the work of Reddington et al. [26], who prepared a combinatorial electrode array of different elemental compositions. Submitting this electrode array to the electrochemical oxidation of methanol, the fluorescent evaluation of the local pH variation was employed to identify the best electro-catalysts for this reaction. A conceptually similar approach has been reported for water splitting electro-catalysts [27].

A more recent innovation by the group of Zhang [28] consists in using EC-FM as a remote reporter of the electrochemical activity, in the sense that the fluorescence modulation is induced at an electrode by the current generated at another electrode where the electrochemical reaction of interest takes place. This strategy has been employed in a slightly modified closed bipolar configuration by Stefano et al. [29] (though not by microscopic intensity measurements) and pushed toward a generalised current-to-optical transductor system by Djoumer et al. [30] (Scheme 1c).

Fluorescent probes
EC-fluorescence requires the use of fluorogenic probes whose fluorescence properties are sensitive to the electrochemical process of interest. These probes are broadly classified into two categories depending on whether these undergo a direct or an indirect electrochemical modulation between the luminescent and non-luminescent state [5,31]. The direct modulation (Scheme 2a) implies that the fluorescent species is itself electroactive and that the fluorescence characteristics depend upon its redox state. The indirect modulation (Scheme 2b) refers to the situation where the fluorogenic probe is sensitive to the chemical environment (e.g., the pH), which is modified by the electrochemical process. The fluorescent species can be thus considered in this latter case as an optical indicator.

Different families of fluorophore and different switching mechanisms are known, and the interested reader is referred to a recent review on the topic [31]. Hereafter, we focus on the specificities of the fluorogenic probes employed in the works described in the previous section.

Direct modulation
Most of the investigation employing the direct modulation of fluorogenic species makes use of molecules belonging to the resorufin (RF) family. This molecule is highly fluorescent and can be converted to the non-fluorescent dihydroresorufin (DH) through an electrochemically reversible reduction. N-acetyl-3,7-dihydroxyphenoxazine, commercially known as Amplex red (AR), is a non-fluorescent protected form of DH. RF can be also generated by the irreversible reduction of the weakly fluorescent resazurin (RZ) which is an N-oxide derivative of RF. The EC-FM behaviour of this resorufin family has been comprehensively described [11,12,32,33]. In recent studies, these compounds are essentially used as remote
optical reporters of an electrochemical reaction. This reaction of interest takes place at an electrode and the generated current induces the electrochemical conversion of the fluorogenic probe at another, reporting electrode, which is either the counter electrode in a classical three-electrode setup [30] or the second pole of a closed bipolar configuration [29]. In either case, the fluorescence modulation at this reporting electrode provides quantitative information (the current at the working electrode in Ref. [30], the concentration of electroactive substance in the remote cell in Ref. [29]) on the electrochemical process taking place at the working electrode. As a direct reactivity visualisation, the oxidation of resorufin red was employed by Sambur et al. [34] to follow in time and space the concentration of Ti–OH⁺ species generated under illumination at a photoanode of TiO₂ nanorods deposited on ITO.

Besides the resorufin family, the direct modulation of anthraquinone-2,6-disulfonate (AQDS) between the fluorescent-reduced state and the non-fluorescent oxidised state was employed to visualise operando transport phenomena taking place in porous carbon electrodes in a typical flow-cell battery configuration [22].

With the aim of comparing the extent of the reaction layer under direct or mediated electron transfer, Ma et al. [13] monitored the fluorescence decrease of 8-hydroxyppyrene-1,3,6-trisulfonic acid (HPTS) upon oxidation.

### Indirect modulation – pH probes

Many electrochemical reactions are coupled with proton transfer steps, leading to spatial variations of the pH around the electrode. Since fluorescent pH indicators have been long known and developed, it is not surprising that the early developments of EC-FM made use of classical indicators such as fluorescein (FL) [8,9,23,24] or quinine [25,26]. The former displays an increase of fluorescence with increasing pH, whereas the latter is more fluorescent in a more acidic environment. Fluorescein remains a popular choice in EC-FM and has been used to image pH gradients generated by electrochemical processes [7,10,16]. Other pH-sensitive fluorescent probes have been reported that present some significant interest for EC-FM.

HPTS, already mentioned, can be a good substitute for FL, because its basic fluorescent form has excitation and emission maxima similar to those of FL, but, unlike this one, it displays only one \( pK_a \) (around 7.2) against two \( pK_a \) (around 4.3 and 6.4) for FL. Both the singly deprotonated and the doubly deprotonated forms of FL are fluorescent (with different quantum yields), leading to a rather poor signal-to-noise ratio since only relative intensity variations between “bright” and “very bright” images are recorded. With only one \( pK_a \), HPTS switches between a dark and a luminescent state with increasing pH, providing a higher imaging contrast with all the associated benefits. HPTS has been employed as a remote reporter in Ref. [30] and as a spatial pH indicator in Refs. [14,15]. Incidentally, HPTS has been employed for many years in optical sensors of gaseous CO₂. Spreading a film of HPTS mixed with a hydrated phase transfer agent onto a gas diffusion layer, Zhang and colleagues [35] were able to follow by fluorescence spectroscopy (not microscopy) the transport of CO₂ during its electrochemical reduction at various current densities.

An appealing improvement in pH gradients imaging is the use of ratiometric fluorogenic probes [17,21,27]. Such fluorophores have excitation or emission properties whose dependence on the pH is highly dependent on the wavelength. Typically, the measured intensity at one excitation (or emission) wavelength decreases while it increases at another excitation (or emission) wavelength. In ratiometric measurements, the pH is linked via a calibration curve to the ratio of the intensities collected for the two excitation/emission wavelengths, instead of the intensity measured at only one wavelength. Because the ratio is not proportional to the fluorophore concentration, in contrast to the fluorescence intensity, ratiometric measurements are less sensitive to artefacts, drifts, concentration inhomogeneity, or fluid motion than single wavelength measurements but are more demanding in terms of instrumentation (filter sets, lasers, etc.). The approach is similar to internal standards in analytical chemistry. Examples of ratiometric pH fluorogenic probes employed in EC-FM are \( 2',7'-\text{bis(carboxyethyl)}-5(6)\)-carboxyfluorescein (BCECF) [27], HPTS [17], \( 5(6)\)-carboxynaphthofluorescein (CNF) [21], and LysoSensor Green DND-189 [21]. Combining these two last probes under ratiometric or single wavelength imaging, Fuladpanjeh-Hojaghan et al. [21] were able to map operando pH gradients in the pH range from 1.5 to 8.5.

Another improvement in the visualisation of pH gradient is the use of fluorescence lifetime imaging, as proposed by Lammertink and colleagues [18], with the LysoSensor Green DND-189 fluorescent probe. Lifetime measurements are less plagued with issues such as photobleaching, inhomogeneous fluorophore concentration or excitation intensity and can provide improved spatial resolution.

In a more biological context, Guille-Collignon and colleagues [36–38] have designed specific fluorogenic species, the Fluorescent False Neurotransmitters, which are both pH sensitive in their fluorescence properties and electroactive. These purposefully designed probes enabled them to monitor exocytotic events by coupled fluorescence and electrochemistry with improved spatial and temporal resolution.
Indirect modulation – other probes
While the number of fluorogenic probes undergoing a direct modulation is arguably rather small, there exist numerous indirect fluorogenic probes whose scope is not limited to protons. Whether for the development of optical sensors or biological imaging, numerous fluorescent probes have been designed which are sensitive or even specific to chemical species such as cations, anions, metals, reactive oxygen species, etc. In particular, some interesting probes have been used in the context of electrochemical energy conversion, more specifically lithium batteries. These are discussed, hereafter, though it should be mentioned that not all of them were employed in situ and under electrochemical control.

Qiao et al. [39] have visualised the diffusion of Mn$^{2+}$ ions from a model cathode of LiMn$_2$O$_4$ using as a fluorogenic probe Calcium Green 5N. As inferred from its name, this fluorophore was designed for Ca$^{2+}$ analysis but was found to increase its fluorescence in the presence of Mn$^{2+}$ ions but not of other ions in the investigated system (Li$^+$ and Al$^{3+}$).

A new turn-on fluorogenic probe for Li$^+$ was synthesised by Padilla et al. [40]. The probe, working in the visible range, was found effective to image the diffusion of lithium ions in propylene carbonate.

Traces of water in organic solvents were detected (by in situ spectroscopy, not microscopy) with a newly designed probe made of a non-fluorescent coordination polymer whose water-induced decomposition release water-coordinated fluorescent Tb$^{3+}$ ions [41].

Kostecki and colleagues [42] shed light on the formation of the solid electrolyte interphase of the Li$_x$Ni$_{0.5}$Mn$_{1.5}$O$_4$–carbonate ester system by fluorescence imaging of nickel and manganese metal complexes formed during the battery charge/discharge cycles.

Conclusion
The use of EC-FM is expanding with a large variety of electrochemical systems being investigated, notably electrochemical energy systems. These are characterised by complex interfacial architectures where spatial heterogeneities in the electrochemical reactivities and mass transport phenomena are expected. The works discussed herein show that the clever design of dedicated electrochemical cells combined with the identification or synthesis of appropriate fluorogenic probes makes EC-FM a useful tool for the scientific understanding of such complex systems. Methodological developments are continuously being reported and contribute in establishing EC-FM as a mature technique for the study of electrochemical processes. Given the ever-increasing number of existing selective fluorogenic probes, it is likely that the domain will continue to see new exciting applications of EC-FM in the near future.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Papers of particular interest, published within the period of review, have been highlighted as:
* of special interest
** of outstanding interest

8. A very careful experimental investigation by confocal EC-FM of pH profiles generated by the oxygen reduction reaction, highlighting the role of the buffering conditions and examining practical issues of the coupling.
13. * A confocal implementation of EC-FM for the study of an electroactive enzyme under catalytic conditions.


Imaging by EC-FM reveals transient mass transport behaviours at bipolar electrodes under non-constant electric field.


Design and construction of a dedicated cell for operando visualisation of electrochemical phenomena. Exploiting the spectroscopic properties of fluorogenic species to map the pH in the 1.5 to 8.5 range.


Operando visualisation of the electrolyte transport within the porous electrode of a redox flow battery, using the direct fluorescence modulation of a redox active quinone.


A thoughtful investigation on how to convert EC-FM into a remote electron-to-photon transduction methodology, focusing on the figures-of-merit in terms of time resolution and opening perspectives for the transduction of non-faradic phenomena.


Expanding the use of indirect fluorescence modulation beyond pH imaging. Here, concentration profiles of a metal cation, Mn2+, are reconstructed from fluorescence images. Although not conducted under electrochemical control, the approach is highly relevant for EC-FM.