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Electrochemiluminescence microscopy: from single objects to living cells

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Abstract

Electrochemiluminescence (ECL) is a light-emitting phenomenon triggered by electrochemistry. The resulting optical signal enables spatiotemporal resolution of the luminescent signal that can be imaged with high accuracy. The field of ECL is evolving rapidly from an analytical method widely applied in biosensing to a novel microscopy technique. The efficient generation of ECL depends on the electrode materials, the luminophore/coreactant systems, and experimental configurations, focusing research on improving these parameters. Nowadays, ECL enables the analysis of transient events and micro/nanometric objects, surface boundaries, interfaces and physical phenomena, as well as catalytic and biological processes. Since ECL microscopy offers many novel applications, this article will describe first the context and then the most recent advances in ECL imaging of single objects and living cells or subcellular structures.

Introduction

Electrochemiluminescence (ECL) is a form of luminescence produced by electrochemical means. The resulting light is usually the analytical signal. Moreover, combining the faradaic information (i.e. the current) and the light emission allows to investigate complex mechanisms and processes[1–3]. However, the current is a global measurement of the electron-transfer reactions occurring over the entire electrode surface. The final product of the ECL process is

light and thus it can be resolved spatially and allows imaging spatial heterogeneities, specific catalytic activities, labelled entities or any processes/objects altering or enhancing the ECL emission. Since ECL is initiated by an electrochemical reaction, its intensity reflects also the mass transport properties of the ECL reagents in the reaction layer. These intrinsic features of ECL have been exploited progressively in different configurations for specific applications. In the “positive” mode, the objects under investigation are either labelled with the luminophore or display a higher ECL activity than the electrode itself. The group of Bin Su introduced the “negative” ECL mode[4–6], which is based on the local diffusional hindrance of the ECL reagents by the object studied (e.g. a fingerprint, a cell or an organelle). From a diffusional point of view, it has some similarities with the negative feedback mode of the scanning electrochemical microscopies (SECM). This situation leads in turn to a lower local light emission.

The sensitive optical readout of ECL combined with modern imaging techniques allows single entity analysis and provides usually more information and lower detection limits when compared to classic electrochemical methods. Considering the imaging principle, it is fundamentally different from SECM, which uses an electrochemical probe to scan the surface or object under investigation. A single ECL image contains the whole electrochemical information without the drawback of a scanning probe. In comparison to photoluminescence (e.g. fluorescence), which uses an external light source for the photo-excitation, it may offer a higher signal-to-noise ratio and a lower background. In addition, it alleviates issues of photobleaching and phototoxicity associated with conventional microscopy methods, which requires high illumination powers. Finally, the spatial resolution of ECL microscopy is typically the Abbe’s diffraction limit but different possibilities to reach higher resolution at the nanometer scale or even imaging single ECL events have been reported[1,7–9]. Since no excitation light source is required to produce the luminescence, the ECL microscopy setup is relatively simple and the microscopy configuration can be easily adapted to different applications. However, since non-transparent electrodes are very often used, the design of the ECL cell is potentially an issue. Finally, the combination of ECL images with photoluminescence ones allows to acquire complementary information because ECL is based on a different physical principle.

The imaging performances of ECL microscopy depends largely on the mechanisms operating as well as on the experimental configurations. In other words, electrochemistry and chemical reactivity are at the core of the ECL process[10]. The two most widely used ECL systems are: (i) $[\text{Ru}(\text{bpy})_3]^{2+}$ luminophore with tri-*n*-propylamine (TPA) as a coreactant and (ii) luminol or

its derivatives with H₂O₂. Both ECL systems react differently depending on the experimental conditions - if they are freely diffusing or one of them is constrained in the proximity of the electrode - and the comprehension of their mechanisms is essential to develop original ECL microscopies. Another major trend is the use of nanomaterials either as the luminophore (e.g. nanoparticles, quantum dots, etc.) or to modify and nanostructure the electrodes[11–15]. Indeed, nanomaterials offers the possibility to design ECL-based readout strategies with remarkable performances. At the electrode surface level, they are mainly employed to modify the electrochemical interface in order to increase the surface area, which leads to a signal enhancement and more efficient biomolecules binding. Herein, we review some selected examples that are not meant to be exhaustive but they show remarkable recent advances in ECL microscopy, starting from nanostructured materials, to single chemical micro/nano-entities including living cells or subcellular structures, and finally single molecule imaging.

Structuration of the electrode surface

The precise control of the ECL-active (nano)materials and of the electrode surface itself plays a crucial role in the development of high-performance ECL detection and imaging. For example, ITO submicrometric wells electrodes with the walls coated with aluminium were successfully applied for sensitive detection of hydrogen peroxide and glucose[16]. The sensing principle is based on the aluminium electrode material, which is able to re-absorb luminol ECL. Thus, emission originating from diffused species away from the electrode surfaces is suppressed. This approach enables better spatial resolution and opens prospects for super-resolution ECL microscopy. On the contrary, silica membrane accelerates deprotonation and mass transfer of positively charged coreactant and luminophore, increasing the ECL of [Ru(bpy)₃]²⁺/TPA system by two orders of magnitude thanks to its negatively charged ultrasmall channels[17]. The geometry of the cell and the inter-electrode distance in the annihilation mode plays a crucial role. Nanofluidic confinement using electrodes positioned at opposite walls of a 100-nm nanochannel enhances annihilation ECL by shortening the diffusion timescale. The comparison with redox cycling shows different concentration profiles, twice shorter diffusion path of [Ru(bpy)₃]²⁺ and consequently higher currents[18]. Single-atom catalysts induce reactive oxygen species (ROS) production and enhance luminol-based ECL. This can be employed for biosensing[19][20] or to study the ECL mechanism[21] of luminol and its derivatives.

Similarly, in bipolar electrochemistry, the spatial confinement of the potential drop at the level of a micropore induces a locally enhanced polarization of a bipolar object localized in this

region. Gold microelectrodes located in a solid-state micropore are polarized at potentials, which are two orders of magnitude lower than without the micropore. Thus, ECL emission with the model $[\text{Ru}(\text{bpy})_3]^{2+}/\text{TPA}$ system is readily achieved even when applying low driving voltage [22]. Based on a conceptually similar approach, a porous platinum coating on a nanopipette tip confines the voltage drop at the level of the Pt deposit, electrochemically sorting the cytosol into the nanopipette and simultaneously generating ECL of L012 (a luminol derivative operating at pH 7), thus enabling intracellular wireless analysis in vivo[23].

ECL microscopy of single objects

As an electrochemical technique with an optical readout, ECL allows in situ time-resolved analysis of catalytic properties with direct visualisation of catalytic sites at the single-particle level. The imaging of the structure/surface-dependant reactivity of different objects have been reported such as the electrocatalytic activity of 2D gold nanoparticles[24] and nanowires[25] by $[\text{Ru}(\text{bpy})_3]^{2+}/\text{TPA}$ derived ECL. The catalytic activity of Pd tipped, Pd covered and Pd-Au Janus particles[26] and the influence of facet properties of ZnO crystals [27] on catalytic H_2O_2 oxidation in the presence of L012 have been described. Transient ECL events such as nanoparticle collisions on the electrode surface were also detected and imaged. The collision of Pt nanoparticles with an ITO electrode results in current and photon spikes that can be utilised to determine the size and concentration of the nanoparticles due to the difference in electroactivity of both materials[28]. A similar experiment was performed wirelessly in a closed bipolar electrode system, where Pt nanoparticles colliding with a cathode participate in the hydrogen evolution reaction (HER), while ECL generated anodically[29].

A high-resolution ECL imaging of a graphene microsheet was achieved by observing scattered ECL spots. Utilising the stochastic nature of ECL generation and stacking individual images, the intensity of each pixel was used to resolve electrocatalytic properties in the $\text{H}_2\text{O}_2/\text{L012}$ system with 400 nm resolution (Fig. 1)[30]. In contrast, during the HER, the stochastic formation of hydrogen bubbles was used to shut off the luminescence of hollow carbon nitride nanospheres, resulting in ECL blinking on the catalytic sites[31].

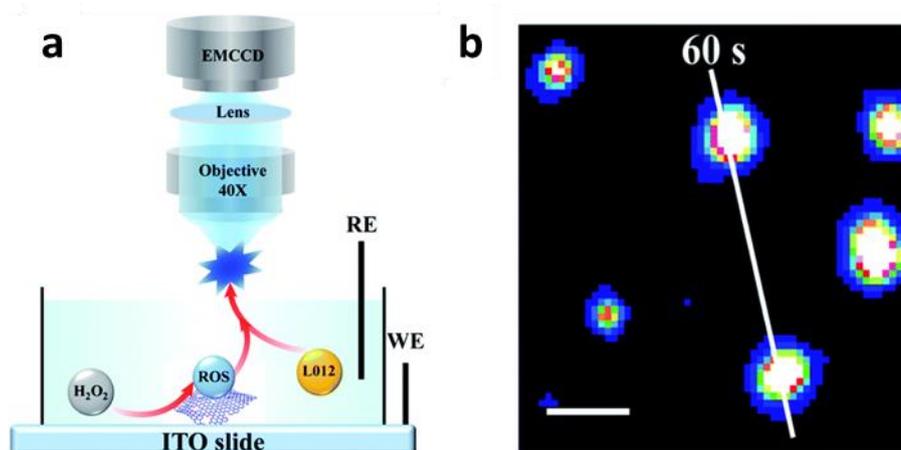


Fig. 1 (a) ECL microscopy setup and (b) ECL images of single graphene oxide microsheets. Scale bar: 5 μm . Adapted from the study of Zhu et al. [30] with permission from the Royal Society of Chemistry, Copyright 2021.

Molecular wires made out of Ir(III) complexes can serve as an efficient ECL emitter and a waveguide that propagates ECL up to distances of 100 μm , with the signal being the brightest at the crystal ends. This approach is promising for the non-invasive study of biological systems[32]. Insertion of carbon nanotubes (CNTs) between $[\text{Ru}(\text{bpy})_3]^{2+}$ labels and the bead used in immunoassay has an electrocatalytic effect and creates a conductive network around the bead, enhancing ECL. CNTs allow simultaneous ECL generation by two mechanisms with the luminophore being oxidised either electrochemically on the CNT or chemically by the freely-diffusing electrogenerated TPA radicals[33].

Apart from being able to visualize catalytic sites, ECL can be used to study phase boundaries, emulsions, droplet collisions and interfacial properties. Even though they can be studied electrochemically[34], the optical output is more informative since it provides a better spatiotemporal resolution[35]. For example, $[\text{Ru}(\text{bpy})_3]^{2+}/\text{oxalate}$ and $[\text{Ru}(\text{dmbpy})_3]^{2+}/\text{oxalate}$ systems in 1,2-dichloromethane/water emulsion were used to estimate the area of the water/electrode interface, the thickness of the phase boundary, the growth dynamics of an oxygen bubble[36] and solvent entrapment[37]. $[\text{Ru}(\text{bpy})_3]^{2+}$ and oxalate are water-soluble, so their ECL signal is constrained in the water phase, while $[\text{Ru}(\text{dmbpy})_3]^{2+}$ is soluble in the less polar organic content, localising ECL at the phase boundaries. Another type of interface between an oxygen bubble and a water phase was imaged by applying anodic overpotentials. These water/gas interfaces are not inert because they accumulate hydroxide ions and lower the

potential needed for their oxidation, thus initiating heterogeneous electrochemical reactions in water[38]. This corona effect was revealed by light emission of luminol. The opening of a giant liposome loaded with $[\text{Ru}(\text{bpy})_3]^{2+}$ and TPA on an ITO electrode was simultaneously followed by amperometry, epifluorescence and ECL microscopy to obtain complementary information on its size, position, open/closed state, as well as the image of efflux material[39].

ECL microscopy of single cells and organelles

In a “negative” ECL configuration, cell adhesions are observed as dark regions hindering the diffusion of ECL reagents[5]. By increasing the concentration of luminophore, long-living $[\text{Ru}(\text{bpy})_3]^{3+}$ formed at the electrode can diffuse and oxidise TPA in the solution, thus expanding the emitting layer and enabling the study of cell-cell junctions[6]. Recently, negative ECL was successfully used to visualise a single functional mitochondrion[40]. It is noteworthy that in this mode, the biological entity is not labelled by the ECL-active luminophore[41]. Therefore, it does not alter the biological activity. A similar imaging strategy was used to assess the influence of environmental factors (electrical stimulation and oxidative stress) on cell morphology[42]. Finally, Xu and coworkers reported a photodynamic therapy driven by ECL. ECL was used in a dual mode: (i) to excite a photo-sensitizer by energy transfer, which produced ROS from O_2 and (ii) to image the resulting gradual morphological changes of the cells[43].

To bypass the need of potentially toxic coreactants and enable the study of dynamic biological processes without labelling, intracellular biomolecules, namely nucleic acids and imidazole in histidine amino acid, were used as coreactants. However, since the luminophore dissolved in the solution cannot pass the cellular membrane, permeabilization or electroporation is required[44]. Another approach relies on the synthesis of new coreactants that enhance the ECL signal[45]. Nitrogen-doped carbon dots modified with a specific peptide serve as a membrane label targeting phosphatidylserine on the membranes of apoptotic cells. They act as a coreactant with $[\text{Ru}(\text{bpy})_3]^{2+}$ in solution, which provides a wider ECL layer and better vertical resolution (Fig. 2)[46]. Tertiary amine conjugated polymer dots were also employed as in situ coreactant-embedded ECL system useful for imaging membrane proteins. This system enables a low applied potential of 1.2V to image non-permeabilized cells with an increased ECL efficiency[47]. Finally, label-free imaging of antigens on the cellular membrane was achieved using the luminol/ H_2O_2 coreactant system’s dependence on the voltage drop caused by binding carcinoembryonic antigens to the cellular surface and applying a square wave voltage[48].

Fundamental research using ECL imaging

The ability to visualise single entities opens new possibilities for unravelling mechanistic and other fundamental properties in the ECL field[49]. For instance, Han et al. used the photobleaching phenomenon on $[\text{Ru}(\text{bpy})_3]^{2+}$ labelled cells to demonstrate that photo- and electro-excitation generate the same excited state[50]. For that, specific regions of the labelled membranes of fixed cells were photobleached confocally and then imaged. ECL microscopy demonstrates a linear correlation between the ECL decrease and the photobleaching of the labels. This approach opens exciting possibilities to study the molecular dynamics at the level of the plasma membranes. They also showed that consecutive anodic pulses lead to ECL signal attenuation of $[\text{Ru}(\text{bpy})_3]^{2+}$ labelled cells[51] and beads[52] due to the lowering of TPA oxidation current, which can be regenerated using an appropriate cathodic treatment.

The measurement of the thickness of the ECL emitting layer (TEL) provides insight into the reaction mechanism[2,53,54] and is an essential parameter for imaging entities on the electrode surface. Changing the buffer capacity affects the rate of TPA^{*+} deprotonation and modulates the concentration profiles of TPA radicals and consequently the TEL in the heterogeneous system [55]. Experimentally, the TEL was obtained using a side-view optical configuration. Nanometer scale vertical resolution was achieved using interference between the ECL emitted directly from the luminophore and the light reflected from the electrode surface[1]. It showed that the TEL of the $[\text{Ru}(\text{bpy})_3]^{2+}/\text{TPA}$ coreactant system in solution varied from 350 nm to 1 μm with the luminophore concentration increasing from 1 μM to 1 mM. This original approach was extended to measure the height and conformation of Ru-labelled molecular linkers attached to the electrode surface. This was consistent with the data obtained by measuring TEL at microtube electrodes, proving the catalytic mechanistic pathway[54]. Paolucci and coworkers[56] reported a highly efficient ECL mechanism close to the electrode surface ($< 1 \mu\text{m}$) that involves a short-living dipropylamine radical and results in an 8-fold enhancement of ECL signal (Fig. 2). This mechanism is emphasized when using branched coreactants like N-dipropylisobutylamine that easily form this radical, improving the signal by 128%.

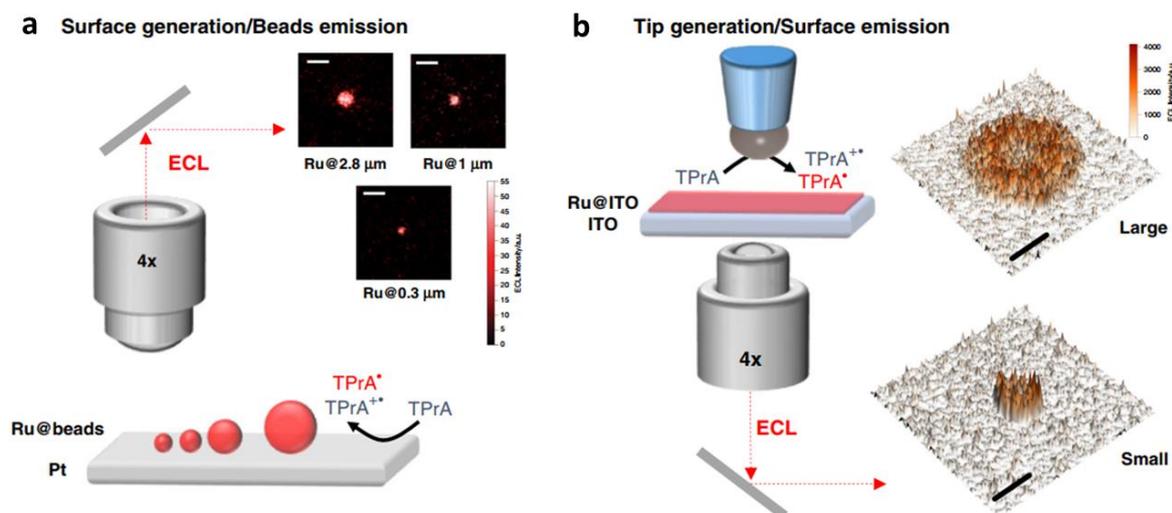


Fig. 2 Spatial distribution of ECL emission from $[\text{Ru}(\text{bpy})_3]^{2+}$ labels in different experimental configurations. (a) The single magnetic beads decorated with $[\text{Ru}(\text{bpy})_3]^{2+}$ were deposited on a platinum electrode surface where an oxidation potential generating ECL was imposed. Scale bar: 5 μm . (b) A microelectrode tip was approached very close to an ITO electrode functionalized by a monolayer of the $[\text{Ru}(\text{bpy})_3]^{2+}$ labels. Scale bar: 500 μm . The oxidation potential was imposed at the microelectrode tip. Adapted from the study of Paolucci et al. [56] with permission from Springer Nature, Copyright 2020.

Single-molecule imaging by ECL

Discrete ECL events in solution were observed for the first time in 1995 by using annihilation of 9,10-diphenylanthracene on an ultramicroelectrode [57]. However, the signal was captured by a photomultiplier tube, and therefore the authors were not able to report any spatial localization of the emitters. In addition, the experiments were performed in drastic experimental conditions using an ultradry organic solvent. Modern ECL applications are exclusively based on coreactant mechanisms that are compatible with analytical measurements in aqueous media at physiological conditions. Feng and coworkers[8] developed a combined optical imaging and electrochemical recording system for monitoring a single molecule by $[\text{Ru}(\text{bpy})_3]^{2+}$ -based ECL reactions in an aqueous solution. They imaged the positions of single photons emitted by individual luminophores involved in ECL events (Fig. 3). To ensure spatial and temporal separation of the light-emitting events, the authors used a dilute solution of the luminophore together with a fine control of the chemical reactivity of the coreactant, which leads to a high dilution of the TPA radicals in the vicinity of the electrode surface. They demonstrated that the collisions of luminophores with the electrode occur randomly by showing statistically that the number of photons emitted follows a Poisson distribution. They applied

their approach to image cells with a resolution as low as 24 nm. Then, the same group applied their strategy to map single photons from individual molecules reacting on Au plates, achieving a resolution of 37 nm [58]. Another ECL approach was reported by Liu and coworkers where single biomolecules were imaged using the enhancement of the signal offered by nanoemitters formed by $[\text{Ru}(\text{bpy})_3]^{2+}$ -doped silica/Au nanoparticles. Single protein molecules were first imaged on the electrode surface and then on the cellular membrane[9].

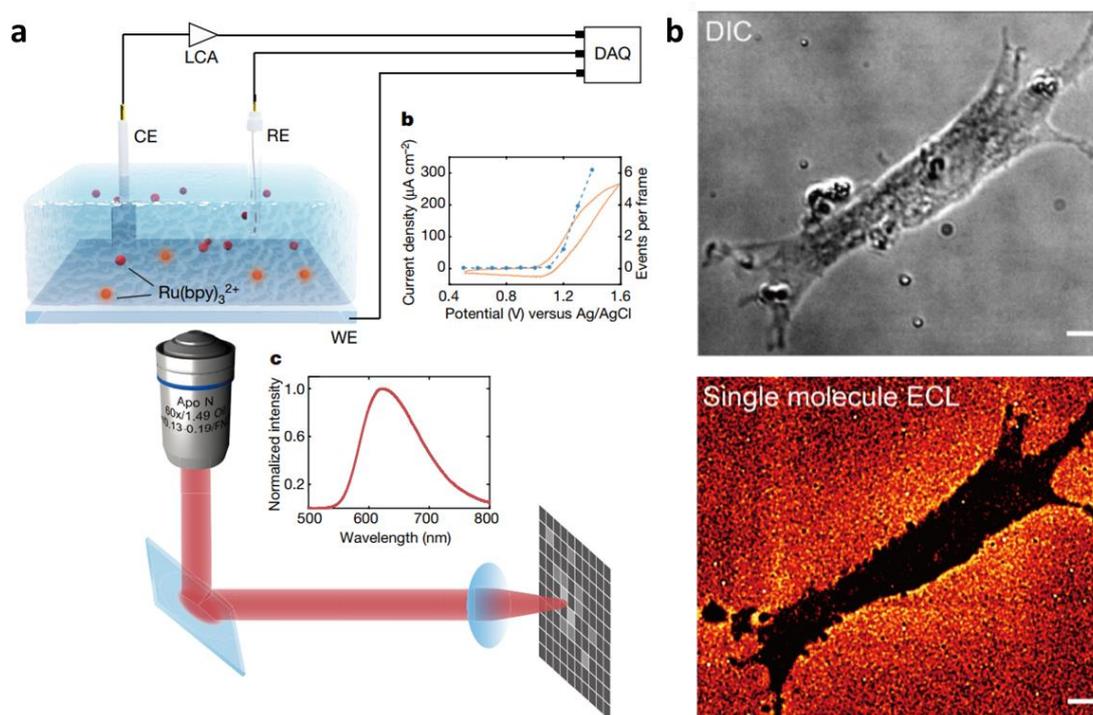


Fig. 3 (a) Single-molecule ECL microscopy setup and (b) imaging of a PC12 cell by differential interference contrast (DIC) and ECL microscopy. This image is reconstructed from 15,000 frames with exposure time of 10 ms. Scale bar: 5 μm . Adapted from the study of Feng et al. [8] with permission from Springer Nature, Copyright 2021.

Conclusions

Electrochemiluminescence is presently a very active field of research. Apart from being a powerful analytical technique, the optical readout opens a wide range of applications in microscopy. Indeed, ECL offers key advantages such as its sensitive optical output, spatial and temporal resolutions, thus allowing the imaging of interfaces, discrete events in a solution, single cells, organelles, molecules and dynamic biological processes. Coupling ECL with microscopy opens possibilities to remotely study biological processes and observe discrete events, while the ECL can simultaneously be used as a light source in photodynamic therapy or

other resonance energy-transfer processes. With ongoing improvements of the electrode materials, luminophores, understanding of the ECL mechanisms, and development of original configurations, the sensitivity and resolution of the technique have reached super-resolution ECL imaging, and even single molecule mapping very recently. We envision that ECL microscopy will continue to follow 4 main trends: (i) imaging of single entities or processes; (ii) coupling with more sophisticated optical techniques; (iii) investigation of the (electro)chemical reactivity of single molecules and (iv) study of more complex biological samples and processes. Indeed, the imaging of single ECL events in solution provides new methods to unravel the reaction mechanisms and kinetic properties in a (bio)chemical system. It broadens the possibilities for its application and consequently the scope of this dual electrochemical/optical method.

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Declaration of interest: none

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

• Paper of special interest

•• Paper of outstanding interest

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Declaration of Interest Statement

Declaration of interest: none