Analytical and Bioanalytical Chemistry (2019) 411:4365–4374
https://doi.org/10.1007/s00216-019-01734-0

TRENDS

Electrochemical monitoring of reactive oxygen/nitrogen species and redox balance in living cells

Marco Malferrari[1] · Maila Becconi[1] · Stefania Rapino[1]

Received: 18 December 2018 / Revised: 19 February 2019 / Accepted: 27 February 2019 / Published online: 23 April 2019

Abstract

Levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells and cell redox balance are of great interest in live cells as they are correlated to several pathological and physiological conditions of living cells. ROS and RNS detection is limited due to their spatially restricted abundance: they are usually located in sub-cellular areas (e.g., in specific organelles) at low concentration. In this work, we will review and highlight the electrochemical approach to this bio-analytical issue. Combining electrochemical methods and miniaturization strategies, specific, highly sensitive, time, and spatially resolved measurements of cellular oxidative stress and redox balance analysis are possible.

Keywords Electrochemical techniques · Reactive oxygen species · Reactive nitrogen species · Cellular redox balance · Living cells · Ultramicroelectrodes

Introduction

Electrochemical methods have been increasingly employed in bio-analytical applications thanks to (i) their high sensitivity and selectivity, (ii) the possibility to perform quantitative measurements of different reactive species at the same time, and (iii) the high time and space resolution of the electrochemical measurements which use miniaturized tools. It is possible to analyze cellular environment and to monitor the cell response to a precise stimulus; furthermore, extracellular and intracellular analysis, in multi-cellular and single-cell configuration, can be performed. Miniaturizing tools is mandatory to elucidate the role of single entities in the whole system and to provide a better insight into the cellular phenomena. Electrochemical methods preserve the cell viability and allow for a real-time investigation of living systems.

The role of reactive oxygen and nitrogen species (ROS and RNS, respectively) in biology has been widely studied in the past [1, 2] and it is still a very hot topic. Several ROS and RNS molecules have been shown to be generated in living cells, both as a consequence of specific protein activities or unwanted events resulting from pathological conditions [3]. In virtue of their high reactivity, the knowledge regarding their fate and role in living systems is continuously updated and new aspects about their production and regulation in the cell physiology are constantly emerging in the literature [4, 5]. The redox homeostasis is really important in cellular physiology and its unbalance plays a central role in human diseases, such as neurodegenerative diseases [6], cancer [7, 8], and heart failure [9]. However, ROS are involved also in normal cell signalling [2, 10]; several observations suggest that ROS and RNS in certain amounts and produced for well-defined times work as signalling molecules in several not-pathological cell processes, e.g., stem cell self-renewal and differentiation [11], neural differentiation [12, 13], and cell proliferation [14]. Furthermore, the fundamental role of ROS in the immunological response and inflammatory and injury healing processes is well known.

In the bio-analytical chemistry field, great attention has been paid for the development of methods able to detect such biological relevant species and to evaluate the redox homeostasis of cells. In this article, the potentialities of the electrochemical methods to address this challenge will be highlighted. High-spatially and time-resolved electrochemical measurements of ROS and RNS and of cellular redox balance will

Published in the topical collection *Young Investigators in (Bio-)Analytical Chemistry* with guest editors Erin Baker, Kerstin Leopold, Francesco Ricci, and Wei Wang.

✉ Stefania Rapino
stefania.rapino3@unibo.it

[1] Department of Chemistry "Giacomo Ciamician", University of Bologna, Via F. Selmi 2, 40126 Bologna, Italy

be reviewed, also in comparison with other commonly used methodologies. As a matter of comparison with electrochemical methods, bio-analytical techniques commonly used to monitor ROS and RNS will be briefly introduced at the beginning of the manuscript. We will then present electrochemical investigations of ROS and RNS production by populations of cells, single cells, and purified mitochondria with high temporal resolution, selectivity, and sensitivity. After that, the use of such developed tools in configurations allowing for spatial resolved investigation will be reviewed: scanning electrochemical microscopy measurements that allowed imaging of ROS and RNS production and of redox state will be presented, and some foundational results arising from this field will be highlighted. Finally, we will discuss the advancements in the development of electrochemical nanotools as enabling technologies to investigate intracellular production of ROS and RNS in living single cells. We will report on the results in this context, which allowed for the real-time and selective investigation of intracellular ROS and RNS levels without threatening the cell viability.

Pro and contra of non-electrochemical techniques employed to measure reactive species in cellular systems

Fluorescence microscopy, electron paramagnetic resonance, and genetic probes [15, 16] are the bio-analytical approaches, not based on electrochemistry, usually employed. HPLC, LC-MS, and mass spectrometry [17, 18] have been also used, mainly to investigate extracellular production of ROS.

The high, complicated reactivity of fluorescence probes has been largely reviewed [19] and their specificity debated [15, 16, 19]; furthermore, they can promote formation of secondary reactive species by redox cycling. Low information can be obtained on the time course of ROS and RNS, while their reaction products or unwanted by-products accumulate in the cellular system. However, these probes are easy to use and often can be targeted to specific intracellular compartments. Relevant progress towards specificity has been made with boronate fluorescent probes [20], which have been reported as highly specific for hydrogen peroxide detection.

EPR techniques are recognized to be the most specific methods for ROS and RNS identification in cellular systems, but this technique suffers from complex and expensive experimental equipment.

Genetic probes have been extensively developed in the last 20 years [21]; targeting to different cell compartments and good specificity for specific ROS and RNS is possible nowadays. However, the documented impact in most cases of genetic probes on cell redox homeostasis, their time-consuming production, and the requirement of specific competencies restrict their wide employment.

It should be also mentioned that all the cited probes are essentially antioxidants and their reactions with ROS and RNS species intrinsically decrease their amounts in the cellular environment, perturbing the system under investigation.

In the following, we will describe how the electrochemical methods face all these aspects and the reasons why we believe the electrochemical monitoring of these reactive species will grow in importance and diffusion.

Electrochemical probes for ROS and RNS detection in isolated mitochondria and entire cells

The first measurements of ROS and RNS production by living cells accomplished with electrochemical methods regarded the extracellular release of reactive species. The unique electrochemical approach allowed for the detection of very small amounts of substance by sensing the thin extracellular space in close proximity to the cell membrane. The single-cell release of ROS and RNS in the extracellular space, in most cases as a consequence of mechanical stimuli of cell plasmatic membranes [22], has been investigated by using an artificial synapsis configuration, i.e., bringing in close proximity to the cell surface the microelectrode (Fig. 1a, panels A and B); carbon microelectrodes were modified with nanoporous black platinum [29, 30] to enhance the sensibility towards ROS and RNS via amperometric detection. Correlation between the mechanical stimuli and the activation of cellular NADPH oxidase accounted for these large, extracellular production of reactive species from human fibroblasts [31]. The production of ROS and RNS was studied in murine macrophages, both upon mechanical or immuno stimuli [23, 32] and in several human cell types, e.g., xeroderma pigmentosum human cells [33], human fibroblasts [34], and human breast cancer cells [35]. These studies returned informative insights on the amount of oxidative species produced by these cellular lines and on the type of the involved oxidative species, e.g., hydrogen peroxide, nitric oxide, and peroxynitrite, by measuring at different working potentials [24] (Fig. 1a, panel C).

Release of nitric oxide from single human endothelial cell (T-HUVEC) has been reported [36]; specific detection of this compound was achieved by modification of platinum microelectrodes by electrodeposition of nickel-tetrasulfonate phthalocyanine tetrasodium salt on electrodeposited platinum clusters. Higher selectivity for nitric oxide, in respect to negatively charged compounds, e.g., NO_2^-, was obtained by coating the modified electrode with Nafion membranes; the authors were able to measure the time course of bradykinin-stimulated release of nitric oxide from a single endothelial cell.

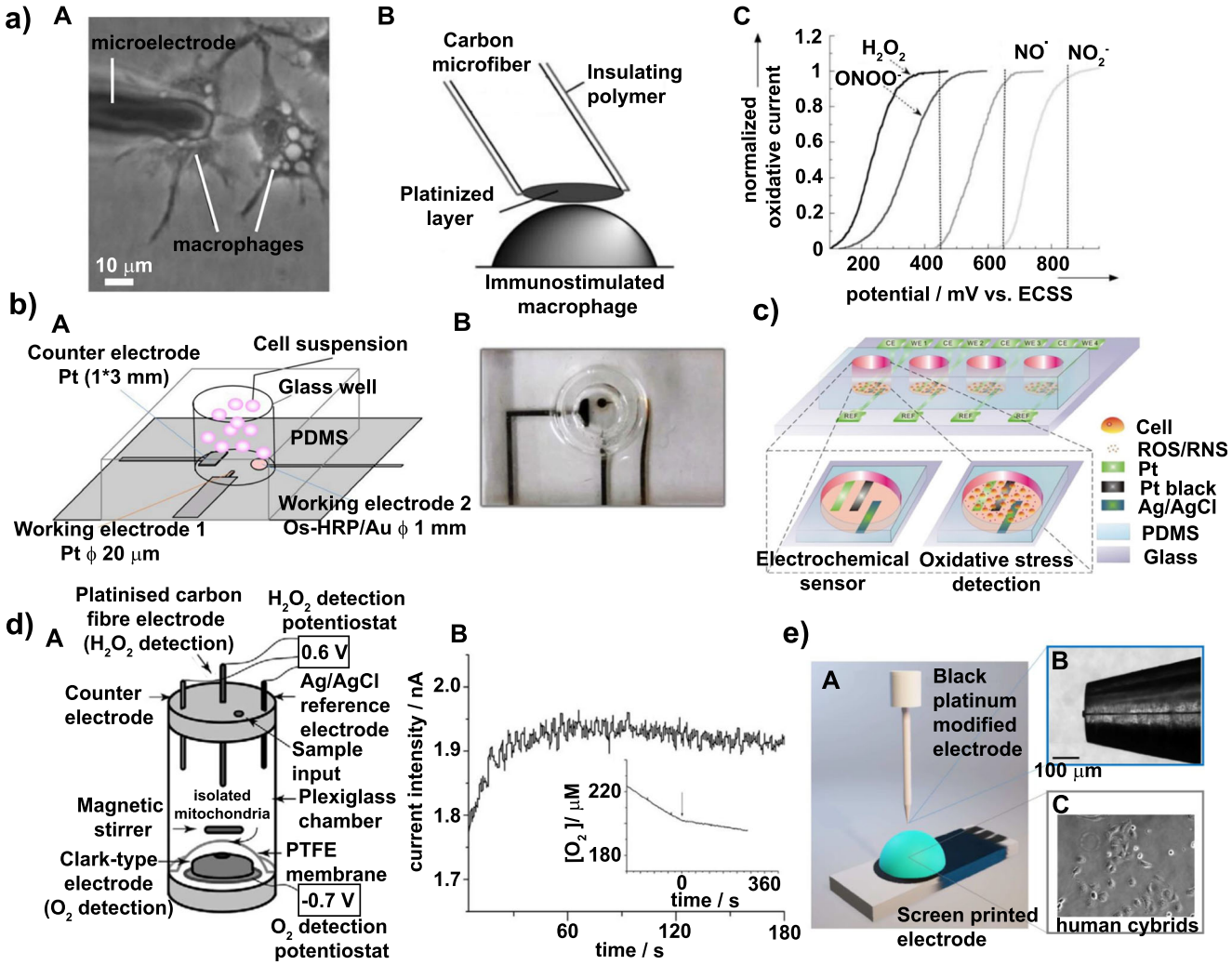

Fig. 1 Electrochemical methods to measure ROS and RNS from single cells, cell populations and suspensions of purified mitochondria. (**a**) Phase-contrast microscopy image (**A**) and scheme (**B**) of the artificial synapsis configuration employed to measure ROS and RNS release to the extracellular space from a single macrophage induced by immunostimulation. (**C**) Normalized voltammograms obtained in the presence of H_2O_2, $ONOO^-$, NO, and NO_2^- with black platinum carbon microelectrodes. Images (**A**, **B**, **C**) were adapted with permission from ref. 23, 24. Copyright © 2008, 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (**b**) Representation (**A**) and image (**B**) of chip biosensor for simultaneous measure of oxygen consumption and H_2O_2 production from THP-1 human monocyte and HL-60 promyelocytic leukemia cells upon immunostimulation. Adapted with permission from figure as originally published in ref. 25 (doi: https://doi.org/10.3389/fphys.2016.00109). (**c**) Scheme of the multichamber device for simultaneous detection of ROS and RNS release from cell populations. Adapted with permission from ref. 26. Copyright © 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (**d**) (**A**) Schematic representation of the integration of a black platinum electrode in an oxygraph apparatus for the simultaneous measure of H_2O_2 and O_2 in suspension of purified mitochondria. (**B**) Time course of hydrogen peroxide production and oxygen consumption (see inset) from purified mitochondria as a consequence of inhibition of respiratory complex III with antimycin. Adapted with permission from ref. 27. Copyright © 2012 Elsevier B.V. (**e**) (**A**) Scheme of the three electrode apparatus employed to measure hydrogen peroxide production upon activation of respiratory complexes in cellular models of mitochondrial diseases. (**B**) Phase-contrast microscopy image of the black platinum working microelectrode. (**C**) Cellular models of mitochondrial diseases. Adapted with permission from ref. 28. Copyright © 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Simultaneous amperometric measurement of oxygen consumption and H_2O_2 production during respiratory burst from populations of THP-1 human monocyte and HL-60 promyelocytic leukemia cells induced by phorbol 12-myristate 13-acetate (PMA) was obtained with chip biosensors [25] (Fig. 1b). The authors employed a platinum microelectrode to selectively measure oxygen and gold [25] or indium tin oxide [37] electrodes, modified with horseradish peroxidase (HRP) embedded in a polymeric matrix, to reduce hydrogen peroxide with osmium $Os^{+/2+}$ as the redox mediator [37, 38].

Production of ROS and RNS from a murine macrophage population was compared to that measured in single cells by employing a multichamber device equipped with a three-electrode configuration and black platinum as a working electrode [26] (Fig. 1c). This configuration, developed to detect in parallel several oxidative species and to diminish, through

parallelization, the amount of measurements needed to reach statistical significance, allowed the comparison of ROS and RNS amounts (i.e., H_2O_2, NO, NO_2^-, $ONOO^-$) produced from single cells and from population of murine macrophages as a consequence of stimulation with a calcium ionophore. Lately, this approach was integrated with a microfluidic device for aerobic culture of cells and downstream detection of ROS and RNS produced from the cell population [39].

Modified microelectrodes were applied also for the detection of ROS produced from bacterial populations. Platinum microelectrodes, modified with multiwalled carbon nanotubes embedded in an ionic liquid (pyridinium hexafluorophosphate-based) conducting matrix, were shown to gain good sensibilities for H_2O_2, i.e., from 250 nM to 5 mM [40]; this sensor was used to characterize metabolic hydrogen peroxide production from a biofilm of bacterium *Streptococcus gordonii*.

Mitochondria are the main physiological source of ROS [41], and as a consequence, the generation and regulation of superoxide and hydrogen peroxide from these dynamical organelles have been intensively studied [42]. Several authors employed electrochemical methods to detect ROS and RNS from mitochondria. We used a black platinum-modified carbon microelectrode integrated in an oxygraph apparatus to measure in parallel the time courses of ROS production and oxygen consumption from purified mouse liver mitochondria [27] (Fig. 1d). This study showed ROS production under different regimes of activation/inhibition of the four respiratory complexes and allowed for correlation with the rate of oxygen consumption from oxidative phosphorylation.

H_2O_2 production upon respiratory chain activation has been studied employing platinum microelectrodes modified with HRP embedded in a polymeric matrix to reduce hydrogen peroxide with mobile osmium $Os^{+/2+}$ as the redox mediator [43], an approach similar to that used previously to specifically detect H_2O_2 with gold and indium tin oxide electrodes in cell populations [37]. The authors succeeded in extending the sensibility of electrochemical specific H_2O_2 detection for five orders of magnitude, from few nanomolar up to approximately 100-μM range. The HRP-based sensor was used to measure hydrogen peroxide production upon respiratory chain activation in suspensions of purified mitochondria, suggesting different regimes of ROS production during oxygen respiration.

Recently, we coupled black platinum microelectrodes to screen printed electrodes to quantify ROS production upon respiratory chain activation in cellular models of mitochondrial diseases [28] (Fig. 1e). Partial permeabilization of cell plasmatic membrane with digitonin allowed to quantify and compare H_2O_2 production in cellular models of two mitochondrial diseases due to specific defects on respiratory complexes (a microdeletion of 6 amino acids on complex III, which leads to a multisystem disorder and a pathogenic mutation on ND1 subunit of complex I, which causes Leber's hereditary optic neuropathy). The small volume of the analyzed cell suspension, restricted by the use of the drop configuration of the screen-printed electrode, requires for a relatively low amount of cells (1×10^6). This approach paves the way to the possibility of investigating and quantifying, in entire cells, the role of each respiratory complex in the pathological and physiological production of ROS.

The studies presented so far contributed to elucidate the amount, typology, and causes of ROS and RNS production in living cells; as depicted in the next chapter, such tools used as probes for electrochemical imaging techniques were employed to spatially image the concentrations of these species and the overall cellular redox state, resolving the involved processes in the space at the single-cell level and revealing the actual heterogeneity within a population of cells.

Electrochemical imaging of ROS and of the cellular redox state

Great attention has been devoted to the possibility of spatially resolving the information enabled by the use of ultramicroelectrodes and microchips [44]. Scanning electrochemical microscopy (SECM) allows for an effective exploitation of the unique properties of ultramicroelectrode (UME). In SECM, the movement and the position of UMEs are finely controlled by the use of stepper motors and piezoelectric components. UMEs are scanned in proximity of a sample while recording a faradaic current, which depends on both the topography and the functional activity of the sample itself [45–47].

SECM has enabled the non-invasive and quantitative analysis of intracellular redox activity by using mediators that shuttle electrons between the microelectrode and the inner cell environment. In particular, hydrophobic mediators are known to permeate the cell membrane, diffuse in the intracellular domains, react with the intracellular redox compounds, and report back to the electrode the cellular redox state.

The first application of SECM to probe the redox activity of cells was reported by Prof. Mirkin's group [48]. The intracellular redox state was investigated, non-invasively, by measuring the rate of transmembrane charge transfer (Fig. 2a). Menadione and naphthoquinone redox species were used to probe the redox reactivity of different cell types: non-transformed human breast epithelial cells, engineered MCF10A cells for the overexpression of protein kinase Cα (PKC α), and metastatic MDA-MB-231 human breast cells. Non-motile and motile cells were reported to exhibit statistically relevant differences. In 2003, Feng et al. used a similar approach to distinguish between cancer and normal cells by their redox activities [50]; maps of both homogeneous and heterogeneous mix of normal and metastatic human breast cells were obtained by SECM imaging and they were compared to optical and fluorescent images (Fig. 2c).

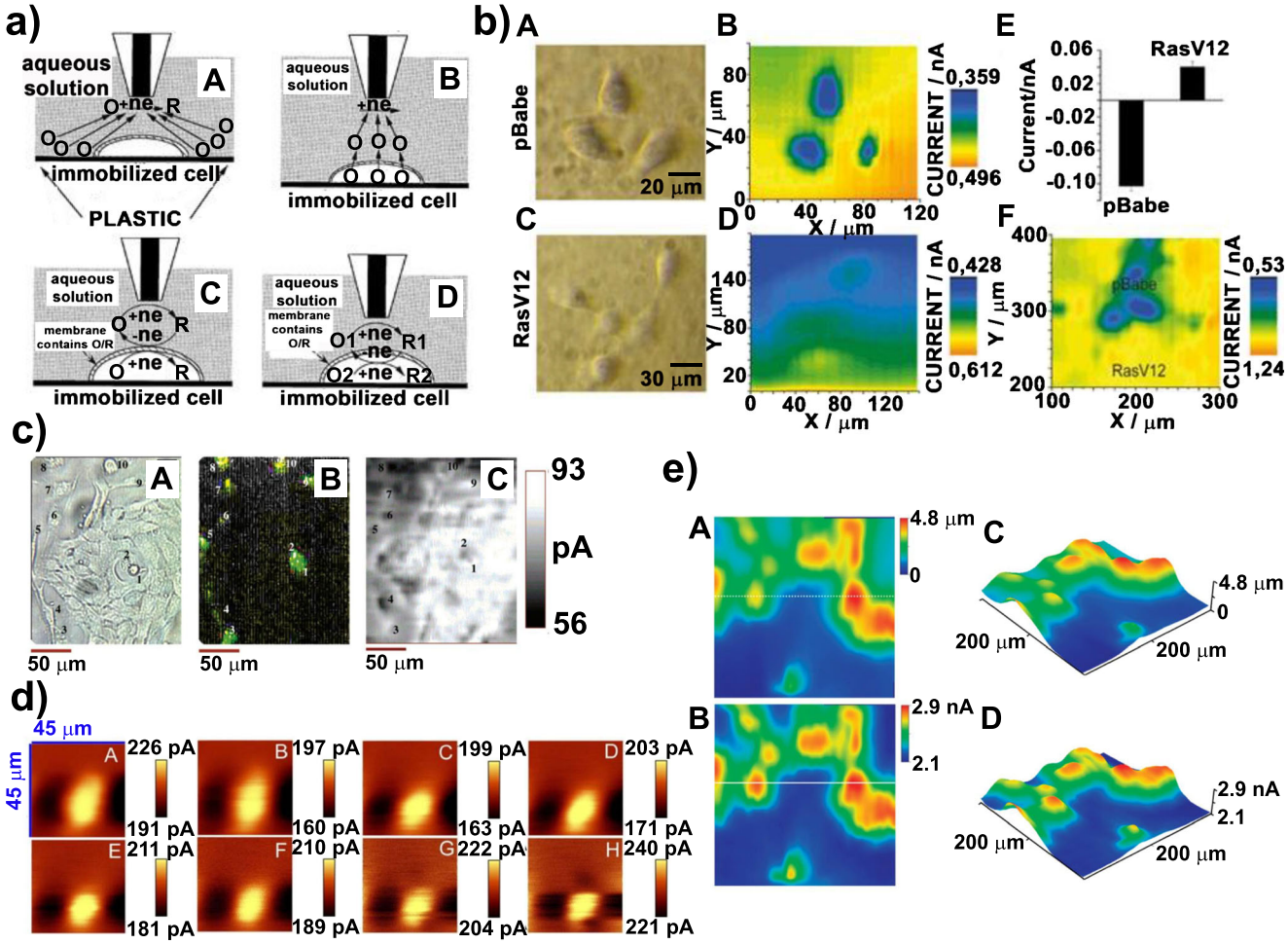

Fig. 2 Scanning electrochemical microscopy (SECM) of the redox state of living cells. (**a**) Schematic representation of SECM experiment with oxidized/reduced (O/R) redox mediator able to permeate (**B**, **C**, **D**) or not (**A**) cell plasmatic membrane. When approaching the surface of the cells, negative feedback is observed for redox mediators which does not permeate into the cell, due to hindered diffusion of the species to the electrode. Increased currents are obtained when: (**B**) due to the diffusion of the redox species from cell cytoplasm to the extracellular matrix; (**C**, **D**) redox mediator in the extracellular space is regenerated through the same redox species which diffuses to the cell cytoplasm or from reducing/oxidizing cellular moieties, respectively. Images adapted from ref. 48. Copyright (2000) National Academy of Sciences, USA. (**b**) Transformation of MCF10A human breast cell lines with RasV12 oncogene shifts cell redox homeostasis to more reducing values, as showed by SECM images with ferrocene-methanol as redox mediator of RasV12 MCF10A (**B**) and MCF10A pBabe control cells (**D**). Phase-contrast microscopy images (**A**, **C**) of cells showed in (**B**, **D**). Panel (**E**) shows average quantification of currents recorded from 10 RasV12 and 10 pBabe cells. SECM image of a coculture of RasV12 and pBabe (**F**) highlighted differences in redox homeostasis as a consequence of oncogene transformation. Images adapted from ref. 49. Copyright © 2015 Elsevier B.V. (**c**) Coculture of MDA-MB-231 and MCF10A cells: transmitted ligth, (**A**); fluorescence signals coming from cell aspecific labeling, (**B**); SECM image using menadione as redox mediator, (**C**). Adapted with permission from ref. 50. Copyright © 2003, American Chemical Society. (**d**) Hydrogen peroxide SECM image of T24 cells stimulated with heat-inactivated *Escherichia coli*. (**A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**) represent successive times (0, 16, 32, 47, 65, 79, 97, and 115 min) following exposure to inactivated bacteria. Adapted with permission from ref. 51. Copyright © 2010 Elsevier B.V. (**e**) Bidimensional (**A**, **B**) and tridimensional (**C**, **D**) SECM images of topography (**A**, C) and hydrogen peroxide and oxygen (**B**, D) released from RAW 264.7 cells. Adapted with permission from ref. 52. Copyright © 2010, American Chemical Society

Electrochemical monitoring of intracellular NAD(P)H:quinone oxidoreductase (NQO) activity was reported for single Hela cells from Prof. Matsue's group. They used SECM and a double redox mediator approach, namely menadione (that can easily diffuse in the cells) and ferrocyanide (that can be used at higher concentrations than menadione, as it is not cytotoxic, and can be detected more efficiently at the platinum SECM UME) [53].

We employed SECM to evaluate the cancer cell redox state. In particular, in order to quantify redox state associated with normal, in vitro transformed (using the oncogene RasV12), and primary tumor cells, we performed SECM imaging using ferrocene-methanol as the redox mediator (Fig. 2b). Ferrocene-methanol is oxidized at the UME in proximity of the cell membrane, reaches the cell membrane, and equilibrates with the intracellular redox environment, and the pristine ferrocene-methanol is then eventually regenerated. If the aforementioned regeneration is efficient, an increase of the current is measured at the UME. The feedback currents

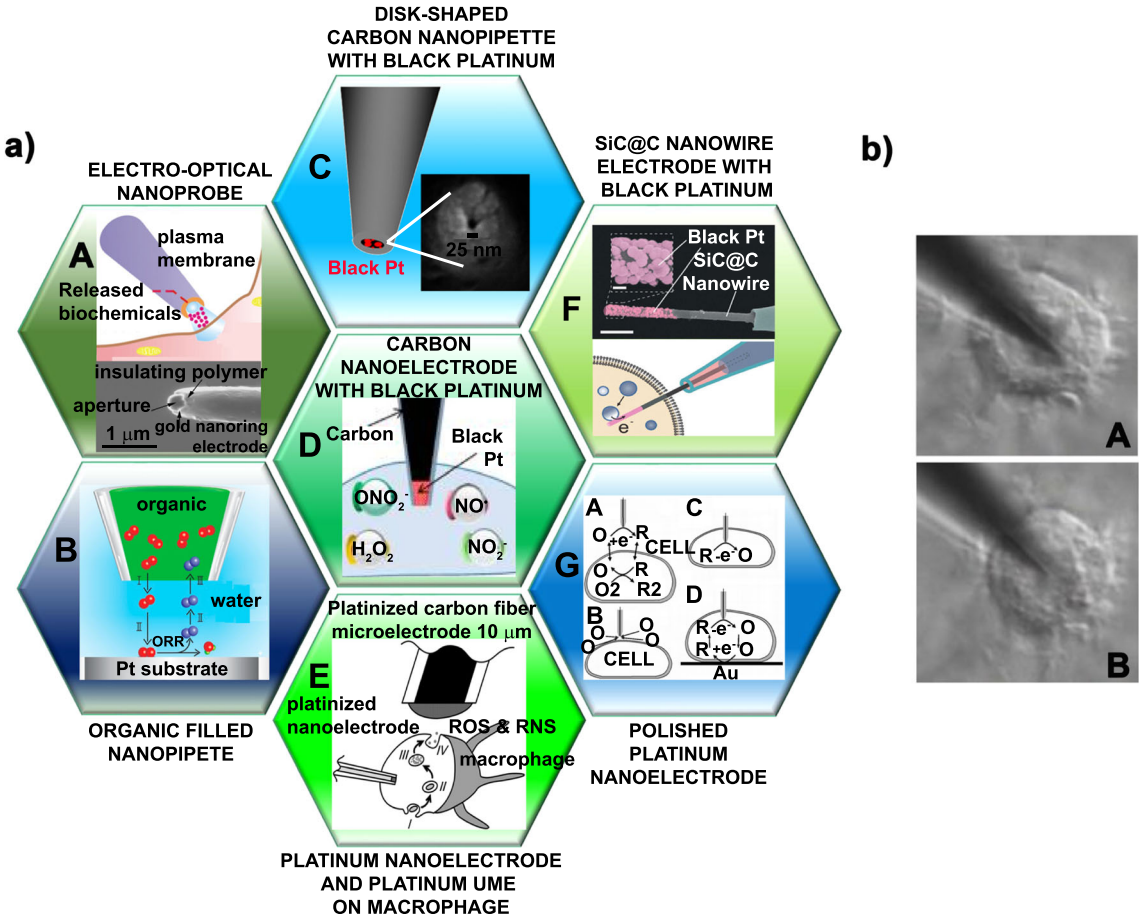

Fig. 3 Nanoelectrodes for extracellular and intracellular measurements of ROS. (**a**) (**A**) Combined amperometric and optical measurements in proximity of the cell membrane with an electro-optical nanotip. Adapted with permission from ref. 57. Copyright © 2011 Elsevier B.V. (**B**) Organic filled nanoprobe to investigate electrocatalytic oxygen reduction. Adapted with permission from ref. 58. Copyright © 2015, American Chemical Society. (**C**) Nanoprobes for brain tissue investigation. Adapted with permission from ref. 59. Copyright © 2014, American Chemical Society. (**D**) Black platinum nanoelectrode for intracellular investigations in human breast cells. Adapted with permission from ref. 60. Copyright © 2015, American Chemical Society. (**E**) Extracellular and cytoplasmatic ROS/RNS measured with ultramicroelectrodes and nanoelectrodes, respectively, in murine macrophages. Adapted from ref. 61. (**F**) Black platinum modiefied silicium-carbon fiber for ROS detection in phagolysosomes. Adapted with permission from ref. 62. Copyright © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (**G**) Extracellular and cytoplasmatic investigations of human breast cell redox state. Adapted from ref. 56. Copyright (2008) National Academy of Sciences, USA. (**b**) Phase-contrast microscopy images of penetration (**A**) and retraction (**B**) of a black platinum-modified carbon nanoelectrode in a cell. Adapted with permission from ref. 63. Copyright © 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

recorded on normal, transformed, and primary cancer cells differ significantly from each other. We hypothesized that the reduction of the oxidized redox mediator operated by the cell was due to an increase of the glutathione levels. We showed an unbalance of the GSH/GSSG ratio in the transformed cells as well as in the primary cancer cells from a lung cancer patient in respect to the normal conditions [49].

Direct electrochemical imaging of ROS produced in cellular systems has been also reported. Single human bladder and kidney epithelial cells were investigated via SECM after exposure to heat-killed uropathogenic *Escherichia coli* GR-12. The cells were imaged by SECM in time-lapse and the cells presenting toll-like receptors were showing prolonged release of ROS (Fig. 2d), while the ones without the receptors did not show ROS production after the exposition to the bacteria. This kind of label-free electrochemical investigation elucidated and can be employed to probe the inflammatory response of cells to gram-negative bacteria [51].

Zhau et al. reported on a study to detect ROS from macrophages; they used an alternating current approach to realize a constant-distance mode and to deconvolve the topographical contribution of the cells. They demonstrated a spatially resolved ROS monitoring and they concluded that the nucleus region of these cells releases more ROS than regions where other organelles are located (Fig. 2e) [52].

Recently, H_2O_2 released from a single monocyte in the extracellular space as a consequence of phorbol ester

stimulation was monitored with a bare platinum microelectrode [54]: the sensibility towards ROS detection of different electrochemical techniques (cyclic voltammetry, linear scan voltammetry, chronoamperometry, and square wave voltammetry) was assessed and their operational ranges compared. The authors employed SECM to map the local amount of H_2O_2 produced following monocyte stimulation by phorbol ester, estimating a time course of hydrogen peroxide production of approximately 1.4 nM/s in the local volume surrounding a single monocyte cell. This ROS production was then compared to the respiratory burst reaction, i.e., immune response of monocytes which consists in the increase of respiratory oxygen consumption coupled to higher ROS production, due to exposure of the monocyte to *Escherichia coli* culture medium, containing several bacterial antigens. Even if a straightforward estimation of hydrogen peroxide was not possible, the increase of hydrogen peroxide in the cell surroundings was estimated to be in the micromolar range.

Hydrogen peroxide was also detected as electrochemical readout of biosensing enzymatic reactions employed at the SECM UME for the electrochemical imaging of lactate release and glucose uptake of human breast epithelial cells [55].

Nanoelectrochemistry and intracellular measurements of ROS and redox balance

The recent developments in the field of nanoelectrochemistry made available new nanotools to be used with systems for their precise positioning. The use of such nanotools increases the spatial resolution of the electrochemical analysis and, at the same time, enables intracellular measurements. In fact, the little dimension of these nanoelectrodes allows for their penetration of cell plasmatic membrane without relevantly affecting cell vitality and without perturbing the intracellular concentration of the species of interests. Some cases of intracellular electrochemical measurements, which represent the absolute state-of-the-art of the field, are herein illustrated.

Nanoelectrodes and nanopipettes are usually characterized by radii about three orders of magnitude smaller than the entire cell dimensions, their use enables the detection in the nanodomains and, at the same time, they have the great advantage of preserving the cell viability when they are used for intracellular measurements [56] (Fig. 3b).

Nanoelectrodes enable real-time, quantitative, and simultaneous measurements of oxidants and antioxidants; furthermore, the small double layer capacitance of the nanoprobes permits sub-microsecond detection allowing for the investigation of fast kinetics bioprocesses [57].

Using such nanoprobes, both extracellular and intracellular nanodomains were explored. The characterization of the environment outside the cells, in close proximity to the cell membrane, is of great interest for the study of cell signalling processes and to evaluate the chemicals fluxes established between intracellular and extracellular regions. Zheng et al. developed a bifunctional electro-optical nanoprobe to measure the local dynamic oxidation activity of a single cell. The nanoprobe they used is an optical fiber with an aperture of 200 nm coated with a 100-nm thickness gold nanoring that is in turn, coated with an insulating polymer (Fig. 3a, panel A). The functionalization of the gold with Prussian Blue confers to the probe the selectivity towards H_2O_2 detection. The thickness of the excitation depth was about 300 nm in the cell membrane thus reducing dye bleaching and background signal and leading to a high spatial resolution. The probe was accurately positioned on the cell membrane with a nanomanipulator and the oxidant production under chemical stimuli was analyzed. Exploiting the double function of the tip, they could record both amperometric and fluorescence signals, and they could correlate the events taking place in intracellular and extracellular compartments. MCF10A, MCF7, and MCF7/HER2 stained with specific fluorescent dyes were investigated in presence or not of the external chemical stimuli. The amounts of reduced thiols were also investigated in these systems in order to monitor the redox balance of these cells. Higher oxidative responses and altered antioxidant levels in cancer cells compared to the normal ones were reported. The authors suggested that the high antioxidant concentration could be correlated with the drug resistance phenomena.

As far as intracellular measurements are concerned, several nanoprobes were developed to penetrate the cell membranes. Sun et al. [56] measured the redox status of single cells with a nanoscale resolution. They reported on the possibility to quantify the oxidative status of the cell by the investigation of the rate of charge transfer across the cell membrane. They performed both extracellular and intracellular experiments on MCF10A cells using platinum nanoelectrodes (Fig. 3a, panel G).

Actis et al. [59] manufactured nanoprobes for SICM-SECM investigation of brain tissue. A disk-shaped carbon nanoelectrode functionalized with platinum (Fig. 3a, panel C) was used to investigate intracellular species. These probes could be precisely positioned inside the neuronal cell minimizing the perturbation to the tissue. A current increase of about 100 fA was recorded upon the penetration of the tissue, which correlated to endogenous intracellular molecules. The authors envisaged the use of these probes to investigate intracellular ROS.

Wang et al. performed intracellular ROS and RNS detection in murine macrophages employing Pt/Pt black nanoprobes [61] (Fig. 3a, panel E). The results of these

experiments show that inside the macrophages the oxidative bursts are shorter in time with respect to the ones recorded outside, in accordance with the hypothesis that the phagolysosomes are able to scavenge the radical species in the cells thus avoiding any damage.

Marquitan et al. [63] developed a carbon nanoelectrode modified with Prussian Blue to selectively and quantitatively detect H_2O_2 in macrophages. Oxidative stress was stimulated by adding H_2O_2 in the medium; it was shown that hydrogen peroxide penetrates the membrane (raise of the intracellular cathodic current) and reaches a stable value corresponding to a steady-state hydrogen peroxide concentration given by the balance between the incoming flux across the cell membrane and the antioxidant scavenging system.

Although oxidative stress is an important carcinogenic factor, a further increase of ROS and RNS concentrations can trigger cancer cell death. Li et al. employed a carbon nanoelectrode modified with black platinum able to detect NO_2^- and H_2O_2 [60] (Fig. 3a, panel D). Intracellular ROS and RNS production has been shown to be higher in cancer breast cells than in normal ones, for which the signal was even too low to be detected. Moreover, upon chemical stimuli (treatment with DAG-lactone), normal cells showed an increase in the oxidative stress due to protein kinase C (PKC) activation.

In order to increase the nanoelectrode mechanical and electrochemical stability, Zhang et al. [62] fabricated a SiC-core shell nanowire electrode (NWE) (Fig. 3a, panel F). These NWEs allow for extensive investigation inside the cell without altering the cytoplasm. Disk-shaped nanoelectrodes are more robust than NWEs, but they do not allow for deep perforation in the cells. Employing this approach, spikes of currents correlated with the ROS/RNS production in the phagolysosomes of the macrophages were reported. The nanoelectrochemistry field is rapidly growing and new well-performing tools are available; in the next future, these probes could be employed for cellular and intracellular ROS/RNS measurements. For example, Zhou et al. reported on nanoprobes used for the determination of the oxygen reduction reaction (ORR [58]), which is central in several energetic fields, e.g., fuel cells, in order to elucidate the reaction pathways and the intermediate species of the electrocatalytic process. They paid special attention to the short-lived intermediates that are not stable in water and are difficult to detect, e.g., $O^{\bullet}{}_2^-$. An electrochemical nanopipette filled with organic phase was built and used to detect at the liquid-liquid interface (Fig. 3a, panel B), the superoxide flux and the radical half-life (that was estimated to be 2 μs). These liquid-liquid electrochemical nanoprobes may be also employed to detect intracellular flux of really short-lived oxygen reactive species. With scanning photoelectrochemical spectroscopy (SPECM), which is a SECM coupled with a light source for the controlled and localized illumination of the sample, Zhao et al. [64] investigate a specific photosynthetic 1 (PS1) protein in order to clarify the applicability of PS1-based photocathodes. They measured the partially reduced oxygen species produced under light exposure of the system. For this measurement, dual Pt microdisk was used, and both O_2 and H_2O_2 were detected at the two microdisks. With this set-up, they gain more information about the electron-transfer pathway at PS1-modified electron surface (PS1/Os-complex). The light irradiation is correlated with the hydrogen peroxide production when the experiment is conducted under aerobic conditions, vice versa biophotodevices operating under anaerobic condition do not produce ROS and thus is reflected in a long-term stability of the system. This same dual electrode configuration may be used to explore the ROS production from single cells and in cell compartments. Finally, sensitive electrochemical probes have been recently developed, based on modification of glassy carbon electrodes with DNA molecules together with mesoporous carbon nitride, gold nanoparticles, and methylene blue [65] or carboxilic acid-functionalized multiwalled carbon nanotubes and gold nanoparticle [66]; the high sensitivity and wide performance range reached envisage the use of these approaches to ROS/RNS detection.

Several strategies for the electrochemical monitoring of ROS, RNS, and cellular redox balance have been reviewed. Limitations are mainly represented by (i) fouling of the electrodes, which often leads to the inability to reuse the same probe for many measures; (ii) the inaccessibility of cell internal compartments, although recently challenged by the use of miniaturized nanoprobes; and (iii) technical difficulties in manufacturing nanoelectrodes.

Outlook

Electrochemical methods present several advantages to address the analytical challenge of ROS/RNS concentrations and redox state detection in living cells: (i) the specificity towards the analyte can be tuned by changing the potential applied to the electrode and by specific electrode modifications (e.g., Prussian Blue, black platinum, functionalized polymers); (ii) no tagging or genetic manipulation of the cells is required; note that the stain accumulation in the cells make it also difficult to monitor the real-time ROS/RNS changes; (iii) the response time of ultramicroelectrodes and nanoelectrodes is in the range of milliseconds and they allow for real-time monitoring of the reactive species production and scavenging; (iv) ROS and RNS are usually located in sub-cellular areas like in specific organelles at low concentration; nano- and microtools allow for the detection of these species in a micro-invasive approach, preserving the cell viability; (v) furthermore, modified microelectrodes combined with a semi-permeabilization of the cell plasmatic membrane can be employed to investigate the time course of ROS/RNS

production and the time changing of the cellular redox balance of entire cells.

For the aforementioned reasons, the authors are confident that electrochemical monitoring of the redox state and of oxidative stress in living cells will establish a new trend in this bioanalytical field. Thanks to the new development in nano- and single-entity electrochemistry, these methodologies will enable a further comprehension of the roles of reactive oxygen species and redox homeostasis, and of the mechanisms governing them, also resolving these phenomena in time and space.

Funding information We acknowledge Associazione Italiana per la Ricerca sul Cancro AIRC (MFAG Id. 19044) for funding.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Sies H. Role of metabolic H_2O_2 generation. J Biol Chem. 2014;289:8735–41.
2. Holmström KL, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol. 2014;15:411–21.
3. Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. Nat Chem Biol. 2008;4:278–86.
4. Shadel GS, Horvath TL. Mitochondrial ROS signaling in organismal homeostasis. Cell. 2015;163:560–9.
5. Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. Mol Cell. 2012;48:158–67.
6. Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov. 2004;3:205–14.
7. Weinberg F, Chandel NS. Reactive oxygen species- dependent signaling regulates cancer. Cell Mol Life Sci. 2009;66:3663–73.
8. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discov. 2013;12:931–47.
9. Brown DA, Perry JB, Allen ME, Sabbah HN, Stauffer BL, Shaikh SR, et al. Mitochondrial function as a therapeutic target in heart failure. Nat Rev Cardiol. 2017;14:238–50.
10. Dickinson BC, Chang CJ. Chemistry and biology of reactive oxygen species in signaling or stress responses. Nat Chem Biol. 2011;7:504–11.
11. Ren F, Wang K, Zhang T, Jiang J, Nice EC, Huang C. New insights into redox regulation of stem cell-renewal and differentiation. Biochim Biophys Acta. 2015;1850:1518–26.
12. Li G, Gong J, Lei H, Liu J, Shawn Xu XZ. Promotion of behavior and neuronal function by reactive oxygen species in *C. elegans*. Nat Commun. 2018;7:13234.
13. Hervera A, De Virgiliis F, Palmisano I, Zhou L, Tantardini E, Kong G, et al. Reactive oxygen species regulate axonal regeneration through the release of exosomal NADPH oxidase 2 complexes into injured axons. Nat Cell Biol. 2018;20:307–24.
14. Diebold L, Chandel NS. Mitochondrial ROS regulation of proliferating cells. Free Radical Bio Med. 2016;100:86–93.
15. Dikalov SI, Harrison DG. Methods for detection of mitochondrial and cellular reactive oxygen species. Antioxid Redox Signal. 2014;20:372–82.
16. Winterbourn CC. The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. Biochim Biophys Acta. 2014;1840:730–8.
17. Zielonka J, Zielonka M, Sikora A, Adamus J, Joseph J, Hardy M, et al. Global profiling of reactive oxygen and nitrogen species in biological systems. J Biol Chem. 2012;287:2984–95.
18. Cheng G, Zielonka M, Dranka B, Kumar SN, Myers CR, Bennett B, et al. Detection of mitochondria-generated reactive oxygen species in cells using multiple probes and methods: potentials, pitfalls, and the future. J Biol Chem. 2018;(26):10363–80.
19. Wardman P. Fluorescent and luminescent probes for measurements of oxidative and nitrosative species in cells and tissues: Progress, pitfalls, and prospects. Free Radic Biol Med. 2007;43:995–1022.
20. Miller EW, Tulyathan O, Isacoff EY, Chang CJ. Molecular imaging of hydrogen peroxide produced for cell signaling. Nat Chem Biol. 2007;3:263–7.
21. Meyer JA, Dick TP. Fluorescent protein-based redox probes. Antioxid Redox Signal. 2010;13:621–50.
22. Amatore C, Arbault S, Guille M, Lemaitre F. Electrochemical monitoring of single cell secretation: vesicular exocytosis and oxidative stress. Chem Rev. 2008;108:2585–621.
23. Amatore C, Arbault S, Bouton C, Dapier J-C, Ghandour H, Koh ACW. Real-time amperometric analysis of reactive oxygen and nitrogen species released by single immunostimulated macrophages. ChemBioChem. 2008;9:1742–480.
24. Amatore C, Arbault S, Bouton C, Coffi K, Drapier JC, Ghandour H, et al. Monitoring in real time with a microelectrode the release of reactive oxygen and nitrogen species by a single macrophage stimulated by its membrane mechanical depolarization. ChemBioChem. 2006;7:653–61.
25. Prasad A, Kikuchi H, Inoue KY, Suzuki M, Sugiura Y, Sugai T, et al. Simultaneous real-time monitoring of oxygen consumption and hydrogen peroxide production in cells using our newly developed chip-type biosensor device. Front Physiol. 2016;7:109.
26. Li Y, Meunier A, Fulcrand R, Sella C, Amatore C, Thouin L, et al. Multi-chambers microsystem for simultaneous and direct electrochemical detection of reactive oxygen and nitrogen species released by cell populations. Electroanal. 2016;28:1865–72.
27. Marcu R, Rapino S, Trinei M, Valenti G, Marcaccio M, Pelicci PG, et al. Electrochemical study of hydrogen peroxide formation in isolated mitochondria. Bioelectrochemistry. 2012;85:21–8.
28. Malferrari M, Ghelli A, Roggiani F, Valenti G, Paolucci F, Rugolo M, et al. Reactive oxygen species produced by mutated mitochondrial respiratory chains of entire cells monitored using modified microelectrodes. ChemElectroChem. 2019;6:627–33.
29. Arbault S, Pantano P, Jankowski JA, Vuillaume M, Amatore C. Monitoring an oxidative stress mechanism at a single human fibroblast. Anal Chem. 1995;67:3382–90.
30. Badets V, Pandard J, Sojic N, Arbault S. Deciphering the platinized surface reactivity to improve the detection of hydrogen peroxide in bioanalyses. ChemElectroChem. 2016;3:2288–96.
31. Arbault S, Pantano P, Sojic N, Amatore C, Best-Belpomme M, Sarasin A, et al. Activation of the NADPH oxidase in human fibroblasts by mechanical intrusion of a single cell with an ultramicroelectrode. Carcinogenesis. 1997;18:569–74.
32. Amatore C, Arbault S, Koh ACW. Simultaneous detection of reactive oxygen and nitrogen species released by a single macrophage by triple potential-step chronoamperometry. Anal Chem. 2010;82:1411–9.
33. Arbault S, Sojic N, Bruce D, Amatore C, Sarasin A, Vuillaume M. Oxidative stress in cancer prone xeroderma pigmentosum fibroblasts. Real-time and single cell monitoring of superoxide and nitric

oxide production with microelectrodes. Carcinogenesis. 2004;25: 509–15.
34. Amatore C, Arbault S, Erard M. Triangulation mapping of oxidative bursts released by single fibroblasts by amperometry at microelectrodes. Anal Chem. 2008;80:9635–41.
35. Lu C, Heldt JM, Guille-Collignon M, Lemaitre F, Jaouen G, Vessieres A, et al. Quantitative analyses of ROS and RNS production in breast cancer cell lines incubated with ferrocifens. ChemMedChem. 2014;9:1286–93.
36. Isik S, Schuhmann W. Detection of nitric oxide from single cells by using constant-distance-mode scanning electrochemical microscopy. Angew Chem Int Ed. 2006;45:7451–4.
37. Inoue KY, Ino K, Shiku H, Kasai S, Yasukawa T, Mizutani F, et al. Electrochemical monitoring of hydrogen peroxide released from leucocytes on horseradish peroxidase redox polymer coated electrode chip. Biosens Bioelectron. 2010;25:1723–8.
38. Vreeke M, Maidan R, Heller A. Hydrogen peroxide and β-nicotinamide adenine dinucleotide sensing amperometric electrodes based on electrical connection of horseradish peroxidase redox centers to electrodes through a three-dimensional electron relaying polymer network. Anal Chem. 1992;64:3084–90.
39. Li Y, Sella C, Lemaitre F, Guille-Collignon M, Amatore C, Thouin L, et al. Downstream simultaneous electrochemical detection of primary reactive oxygen and nitrogen species released by cell populations in an integrated microfluidic device. Anal Chem. 2018;90: 9386–94.
40. Joshi VS, Kreth J, Koley D. Pt-decorated MWCNTs−ionic liquid composite-based hydrogen peroxide sensor to study microbial metabolism using scanning electrochemical microscopy. Anal Chem. 2017;89:7709–18.
41. Murphy MP. How mitochondria produce reactive oxygen species. Biochem J. 2009;417:1–13.
42. Wong HS, Dighe PA, Mezera V, Monternier PA, Brand MD. Production of superoxide and hydrogen peroxide from specific mitochondrial sites under different bioenergetic conditions. J Biol Chem. 2017;292:16804–9.
43. Suraniti E, Ben-Amor S, Landry P, Rigoulet M, Fontaine E, Bottari S, et al. Electrochemical monitoring of the early events of hydrogen peroxide production by mitochondria. Angew Chem Int Ed. 2014;53:6655–8.
44. Lin TE, Rapino S, Girault HH, Lesch A. Electrochemical imaging of cells and tissues. Chem Sci. 2018;9:4546–54.
45. Bartolini L, Malferrari M, Lugli F, Zerbetto F, Paolucci F, Pelicci PG, et al. Interaction of single cells with 2D organic monolayers: a scanning electrochemical microscopy study. ChemElectroChem. 2018;5:2975–81.
46. Rapino S, Valenti G, Marcu R, Giorgio M, Marcaccio M, Paolucci F. Microdrawing and highlighting a reactive surface. J Mater Chem. 2010;20:7272–5.
47. Rapino S, Treossi E, Palermo V, Marcaccio M, Paolucci F, Zerbetto F. Playing peekaboo with graphene oxide: a scanning electrochemical microscopy investigation. Chem Commun. 2014;50:13117–20.
48. Liu B, Rotenberg SA, Mirkin MV. Scanning electrochemical microscopy of living cells: different redox activities of nonmetastatic and metastatic human breast cells. P Natl Acad Sci USA. 2000;97: 9855–60.
49. Rapino S, Marcu R, Bigi A, Soldà A, Marcaccio M, Paolucci F, et al. Scanning electro-chemical microscopy reveals cancer cell redox state. Electrochim Acta. 2015;179:65–73.
50. Feng W, Rotenberg SA, Mirkin MV. Scanning electrochemical microscopy of living cells. 5. Imaging of fields of normal and metastatic human breast cells. Anal Chem. 2003;75:4148–54.
51. Zhao X, Lam S, Jass J, Ding Z. Scanning electrochemical microscopy of single human urinary bladder cells using reactive oxygen species as probe of inflammatory response. Electrochem Commun. 2010;12:773–6.
52. Zhao X, Diakowski PM, Ding Z. Deconvoluting topography and spatial physiological activity of live macrophage cells by scanning electrochemical microscopy in constant-distance mode. Anal Chem. 2010;82:8371–3.
53. Matsumae Y, Takahashi Y, Ino K, Shiku H, Matsue T. Electrochemical monitoring of intracellular enzyme activity of single living mammalian cells by using a double-mediator system. Anal Chim Acta. 2014;842:20–6.
54. Bozem M, Knapp P, Mirceski V, Slowik EJ, Bogeski I, Kappl R, et al. Electrochemical quantification of extracellular local H_2O_2 kinetics originating from single cells. Antioxid Redox Signal. 2018;29:501–17.
55. Soldà A, Valenti G, Marcaccio M, Giorgio M, Pelicci PG, Paolucci F, et al. Glucose and lactate miniaturized biosensors for SECM-based high spatial resolution analysis: a comparative study. ACS Sens 2017; 2: 1310–1318.
56. Sun P, Laforge FO, Abeyweera TP, Rotenberg SA, Carpino J, Mirkin MV. Nanoelectrochemistry of mammalian cells. P Natl Acad Sci USA. 2008;105:443–8.
57. Zheng XT, Hu W, Wang H, Yang H, Zhou W, Li CM. Bifunctional electro-optical nanoprobe to real-time detect local biochemical processes in single cells. Biosens Bioelectron. 2011;26:4484–90.
58. Zhou M, Yu Y, Hu K, Mirkin MV. Nanoelectrochemical approach to detecting short-lived intermediates of electrocatalytic oxygen reduction. J Am Chem Soc. 2015;137:6517–23.
59. Actis P, Tokar S, Clausmeyer J, Babakinejad B, Mikhaleva S, Cornut R, et al. Electrochemical nanoprobes for single-cell analysis. ACS Nano. 2014;8:875–84.
60. Li Y, Hu K, Rotenberg SA, Amatore C, Mirkin MV. Direct electrochemical measurements of reactive oxygen and nitrogen species in nontransformed and metastatic human breast cells. J Am Chem Soc. 2017;139:13055–62.
61. Wang Y, Noël JM, Velmurugan J, Nogala W, Mirkin MV, Lu C, et al. Nanoelectrodes for determination of reactive oxygen and nitrogen species inside murine macrophages. Proc Natl Acad Sci U S A. 2012;109:11534–9.
62. Zhang XW, Qiu QF, Jiang H, Zhang FL, Liu YL, Amatore C, et al. Real-time intracellular measurements of ROS and RNS in living cells with single core–shell nanowire electrodes. Angew Chem Int Ed. 2017;56:12997–3000.
63. Marquitan M, Clausmeyer J, Actis P, Lopez Cordoba A, Korchev Y, Mark MD, et al. Intracellular hydrogen peroxide detection with functionalised nanoelectrodes. ChemElectroChem. 2016;3:2125–9.
64. Zhao F, Hardt S, Hartmann V, Zhang H, Nowaczyk MM, Rögner M, et al. Light-induced formation of partially reduced oxygen species limits the lifetime of photosystem 1-based biocathodes. Nat Commun. 2018;9:1973.
65. Zeng G, Zhu Y, Zhang Y, Zhang C, Tang L, Guo P, et al. Electrochemical DNA sensing strategy based on strengthening electronic conduction and a signal amplifier carrier of nanoAu/MCN composited nanomaterials for sensitive lead detection. Environ Sci Nano. 2016;3:1504.
66. Zhu Y, Zeng G, Zhang Y, Tang L, Chen J, Cheng M, et al. Highly sensitive electrochemical sensor using a MWCNTs/GNPs-modified electrode for lead (II) detection based on Pb^{2+}-induced G-rich DNA conformation. Analyst. 2014;139:5014.