CELL & GENE THERAPY INSIGHTS

CRITICAL ROLE OF AUTOMATION IN THE MANUFACTURE OF CELL & GENE THERAPIES

SPOTLIGHT

EXPERT INSIGHT

Augmenting automated analytics using fluorescent nanosensors

Veeren M Chauhan

Cell and gene therapies (CGTs) are projected to transform healthcare precision in the biotherapeutics sector. However, for their true potential to be realised, advancements must be made to optimising their manufacture, such that CGT production is precise, reproducible and robust. This includes monitoring and control of complex cell culture conditions, such as extracellular and subcellular biochemical parameters, for which there are no readily available automated analytical systems. Biosensors, such as fluorescent nanosensors, provide a tangible solution to augment CGT manufacture, as they enable off-line, online and inline monitoring of the cellular microenvironments. This expert insight highlights how the automated analytical afforded by fluorescent nanosensors, could permit real-time realignment of critical sub-cellular biochemical parameters to enhance CGT manufacture. The insight concludes by evaluating how the integration of fluorescent nanosensors with new and established methods could pave-the-way forward to maximise CGT potential.

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"To measure is to know … If you cannot measure it, you cannot improve it."

–Lord William Thomson Kelvin, 1824–1907.

INTRODUCTION

Measurement aims to quantify every aspect of our surroundings, from the distance to Andromeda, the Milky Way's neighbouring galaxy, to the mass of a Z boson, a subatomic particle in the standard model of particle physics. Standardisation of the seven fundamental measures of length (meter), time (second), mass (kilogram) temperature (kelvin), electricity (ampere), light (candela) and amount (mole)

has enabled the natural sciences and economies to find commonality in measurement units. Order can be established from the apparent disorder through the application of measurement tools to enhance the knowledge of the world we live in. Moreover, the iterative application of new and improved technologies to probe our surroundings has augmented our knowledge and our ability to influence it.

Biological systems, in particular the human body, have attracted the most attention with regard to the human race's drive to understand and influence. Governments, pharmaceutical companies and academic institutions annually invest large sums of money into the development of new technologies and drugs with the aim to enhance longevity or eradicate diseases [1]. However, most of the changes that occur in a biological system, resulting in the development of healthy or diseased tissue, appear in microenvironments at the sub cellular level, which is at the current limit of our understanding. Bearing this in mind there are no readily available shortcuts to effectively manipulate biological systems in a controlled manner, without having some prior knowledge on how they operate. Therefore, to enhance our understanding of the building blocks of life, before strategies are implemented to influence them, sensors or techniques must be developed that are able to map the transport and micro-localisation of critical molecules and ions, which are essential for cellular function.

THERAPEUTIC DISCOVERY

Pharmaceutical companies have attempted to overcome this limit through employment of high throughput automated screening strategies that aim to examine vast numbers of natural and synthetic compounds with the hope they elicit a biological effect. However, this approach was not economically viable for sustained periods, as large amounts of money were invested with diminishing returns, yielding fewer successful drug candidates [2]. However, personalised medicine is changing the way the pharmaceutical industry aims to cure disease. Patients are stratified by characterizing their genetic or biochemical profile to identify the disease etiology. These measurements permit the development of targeted therapy to delivery functional proteins, cells and genes to repair, restore or facilitate the removal disease.

Proteins and viral vectors have been successfully produced using large-scale manufacture systems such as *Pichia pastoris, Saccharomyces cerevisiae, Escherichia coli* or mammalian cell culture. This is because, these systems are well defined and can be monitored effectively using extracellular parameters temperature, pH and dissolved oxygen concentrations, due to the excellent automated feedback. In contrast, cell therapies have been produced for a limited number of conditions. This is due to challenges in optimisation of complex cell culture conditions, which includes a multitude of intricate extracellular and sub-cellular biochemical parameters, for which there are no readily available automated monitoring systems, such that they have proved significantly more challenging to produce. Therefore, for their true potential to be realised advancements must be made

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optimizing subcellular sensory bioprocessing technology, which in turn will augment manufacturing capacity.

SENSORS

Sensors recognise stimuli in their surroundings, to transduce a signal to a detector, which can then be quantified and interpreted as a measurement. Ideally a sensor for biological measurement should:

1. Provide high spatial and temporal resolution, so that dynamic measurements can be made from sub cellular microenvironments;
2. Cause negligible cellular perturbations, such that measurements are independent of the sensing technique; and
3. Demonstrate high sensitivity and selectivity to the analyte of interest, for accurate and precise quantitative measurements.

Paradigm shifts in the development of tools and techniques to manipulate and investigate matter at the micro- and nano-scale, such as scanning electron microscopy (SEM) [3], transmission electron microscopy (TEM) [4], scanning tunnelling microscopy (STM) [5], atomic force microscopy (AFM) [6], scanning ion conductance microscopy (SICM) [7] and optical microscopy [8], have permitted the development of miniaturised sensors, such as optical [9] and electrochemical sensors [10], which can be used to probe the micro-environments found in living cells. Examples of such a technology are optical nanosensors.

Optical nanosensors

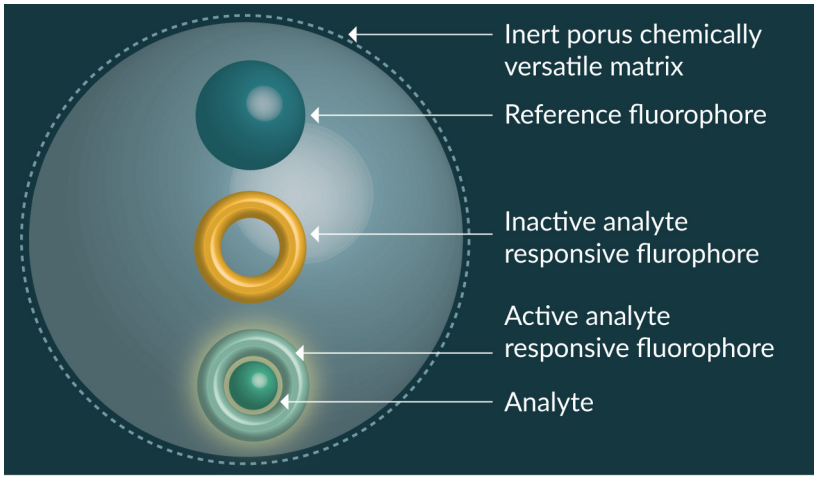
Optical nanosensors are probes that have nanometre-scale sizes in all three dimensions [11] that utilise light in many forms, such as ultra-violet light, visible light, near-infrared and infra-red, to elucidate a vast amount of detail about the inner workings of microenvironments. Of the number of optical nanosensors reported in the literature, probes that utilise the principles of fluorescence and phosphorescence have shown the most promise [12–15]. This is largely due to:

1. The high spatial and temporal resolutions they provide, when imaged with optical microscopes;
2. Enhanced sensitivity, when compared with the weak signals obtained from other techniques such as absorption spectroscopy and Raman scattering; and
3. Can be engineered to be non-invasive and highly specific to analytes of interest.

Due to the diversity of fluorescent sensing elements available, and ability of the versatile nanoparticle matrix to protect the sensing element, development of fluorescent nanosensors has been taken on by several research groups around the world and has permitted the development of ratiometric fluorescent nanosensors (Figure 1). Ratiometric nanosensors typically consist of two or more fluorophores that emit at different wavelengths [16]. There is an indicator fluorophore, which produces a signal in response to the analyte concentration. Whilst a reference fluorophore provides a signal, which is insensitive to the

FIGURE 1

Ratiometric fluorescent nanosensor.



analyte of interest. The combination of fluorophores permits accurate ratiometric measurements to be made. The fluorescence emission can be quantified with the use of analytical techniques such as fluorescence spectroscopy and widefield or confocal microscopy.

Fluorescent nanosensors have been reported for hydrogen ions (pH) [17,18], molecular oxygen [19], calcium [20,21], copper [22], chloride [23], glucose [24,25], iron [26–28], lead [29], magnesium [30], mercury [31], potassium [32], ROS [33–37], sodium [38,39], zinc [40], proteins [41,42], nucleic acids [43,44], ATP [45,46] and temperature [47–50]. The scope for producing new fluorescent nanosensors is limited only by the availability of analyte sensitive fluorophores or receptors that can transduce a signal to fluorescent molecules.

Delivery of nanosensors

Nanosensors have been known to spontaneously cross cell membranes through pinocytosis or phagocytosis. However, not all nanoparticles demonstrate this property. This is because the cellular uptake of nanoparticles is heavily influenced by size shape and charge [51,52]. Therefore a number of strategies have been explored to enhance the delivery in a controlled manner (Figure 2A) [53]. Examples of some of these strategies include pico-injection, gene gun delivery, liposomal incorporation and electroporation.

Pico-injection uses a fine needle to puncture the cell membrane to deliver pico-litres of sample into a single cell (Figure 2B) [53]. This method can cause unwanted cellular perturbations and require a high level of skill to inject a sample into a cell without causing excessive damage. Gene guns have predominantly been used to transfect cell cultures with deoxyribonucleic acid (DNA) [54] and plasmids [55], but their use has also been shown for the delivery of nanosensors across cell membranes [30]. The gene gun blasts dry nanoparticles into a cell culture dish using pressurized helium gas helium as a propellant (Figure 2C) [56]. This method has been found to maintain cell viability whilst delivering large quantities of nanoparticles across cell membranes [57]. It is important to note strategies such as pico-injection and gene delivery are low throughput and may be challenging to incorporate on large-scale autonomous cell production processes.

Liposomal transfection, like gene gun delivery, has also been used to deliver nucleic acids across cell membranes but have shown to be useful at transporting nanoparticles [53]. Liposomal transfection methods utilise artificially prepared vesicles formed from lipid bilayers, which envelop a small volume of

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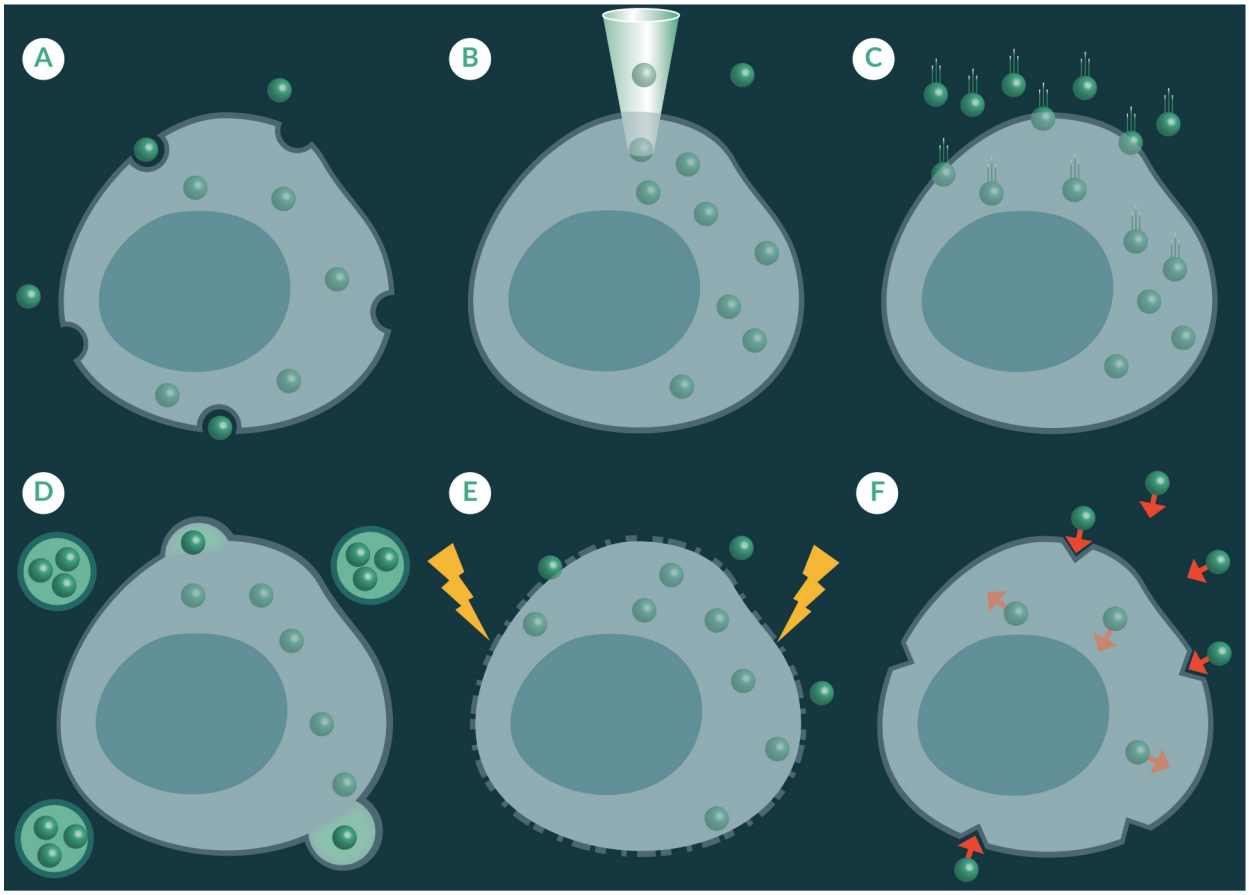
FIGURE 2

Cellular uptake strategies for nanosensors.



Nanosensors can be delivered to cells via **(A)** spontaneous endocytosis, **(B)** pico injection **(C)** gene gun bombardment, **(D)** liposomal fusion, **(E)** electroporation and **(F)** receptor mediated delivery.

nanosensors. The liposomal vesicles fuse with the cell membrane to transfer the contents of the liposome to the cellular cytoplasm (Figure 2D). Whereas, electroporation applies a voltage across a cell membrane to increase its permeability so that foreign material, such as fluorescent nanoparticles, can travel through to sub cellular spaces (Figure 2E), and has been shown to be effective at transporting foreign material across cell membranes of a number of cell lines, including yeast cells [45]. Another approach has been to utilize the versatile nanosensor matrix as a platform to attach chemical moieties that have demonstrated targeted uptake through receptor mediated uptake pathways (Figure 2F). Examples of some of these moieties which have been use to decorate nanoparticles are the universal membrane penetrating peptide trans-activating transcriptional activator (TAT), derived from Human immunodeficiency virus 1 (HIV-1) [58], and the tumour-homing F3-peptide, which is derived from high mobility group nucleosome binding protein 2 (HMGN2) [59].

Due to the number of mechanisms available for nanoparticle delivery, nanosensors have been applied to a range of biological systems including mammalian cell lines, [33,60] stem cells [61], scaffolds for regenerative medicine [62–64] and model

organisms [65,66]. It is important to note targeted placement of probes adjacent to subcellular organelles of interest, such as mitochondria, is an even more challenging. Therefore, subcellular positioning must also be considered if specific bioprocesses are to be monitored [67]. Effective monitoring of critical molecules and ions *in situ* or *in vitro* in model biological systems, such as the ones mentioned above, using fluorescence microscopy could generate diagnostic information biological function and suggest new approaches to enhance cell and gene therapy culture.

INTERGRATING NANOSENSORS FOR CELL & GENE THERAPY

Optimized cell culture encompasses the production of sufficient quantities of viable cells, which have the desired function or therapeutic activity, whilst effectively utilising resources, such as raw materials and time [68]. Therefore, integration of sensors to automatically feedback will enhance cell culture consistency and capacity and concurrently reduce waste of essential resources.

Bioreactors & fluorescent nanosensors

Cell production is typically conducted using bioreactors [69], which are vessels that permit cell expansion by providing the essential culture conditions (e.g., temperature, aerobic/aerobic & pH), nutrients (e.g., carbohydrates, proteins and lipids) and growth factors (e.g., cytokines) [70]. Monitoring of cell culture environments in bioreactors can occur via direct or indirect measurements, as well as offline, online and inline observations [71].

Bioreactors are selected or designed based on the cell culture conditions and required scale of manufacture. Cell culture conditions refers to the ex vivo environments cells prefer to enhance their expansion. For non-adherent cells to homogenise culture conditions this can include the introduction of sheer stress, which can also improve mass distribution of nutrients and growth factors through the culture medium (e.g., orbital shakers, spinner flask and rotating wall vessels) [72]. Whereas rocking [73] or perfusing [74] culture media has also proved effective for homogenization of adherent cells. Hybrid bioreactors that increase the surface area for cell attachment through the introduction of microcarriers [75] and scaffolds [76] that are distributed throughout high sheer environments have also been introduced to improve culture capacity and maximise nutrient and growth factor resource allocation. Scale of manufacture is dependent on ultimate use of cells. Small-scale bioreactors are used for *in vitro* research purposes or clinical allogeneic products in commercial settings, such as chimeric antigen receptor (CAR) T cells [77]. Whereas, large-scale manufacture, which can reach batch sizes of 20,000 liters, are typically used for commercial projects [78].

Direct & indirect measurement

Direct measurement corresponds to whether information on culture parameters is determined on actual measurement of key components (e.g., the partial pressure of oxygen

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or carbon dioxide) in the bioreactor or cells [79]. Whereas indirect measurement corresponds to evaluation of changes in cell cultures marker (e.g., cell surface expression marker and excreted products) after the input parameters have been modified. Therefore, direct measurements permit measurement of real-time events, which can then then be used for automated optimization of cultures. Whereas indirect measurements can provide information of cell behaviour due to changing growth parameters; however, these may include measurement artefacts as a number of intricate changes can affect the ultimate cell output, unless defined biochemical pathways have been identified [80]. Indirect measurements can also be used for real-time optimization of cell cultures, however, as biochemical processes contain an inherent time lag, immediate optimization of growth parameters may be challenging [80].

When fluorescent nanosensors are delivered to subcellular spaces, they permit direct and indirect quantification of biochemical parameters that could be used to for real-time optimization of growth parameters. Direct measurement of biochemical processes is evidenced by measurement of pH in *Saccharomyces cerevisiae* [81], whereas indirect measurement of has been shown by the quantification of hydrogen peroxide in human mesenchymal stem cells [82], a toxic by-product of porphyrin induced photodynamic light therapy.

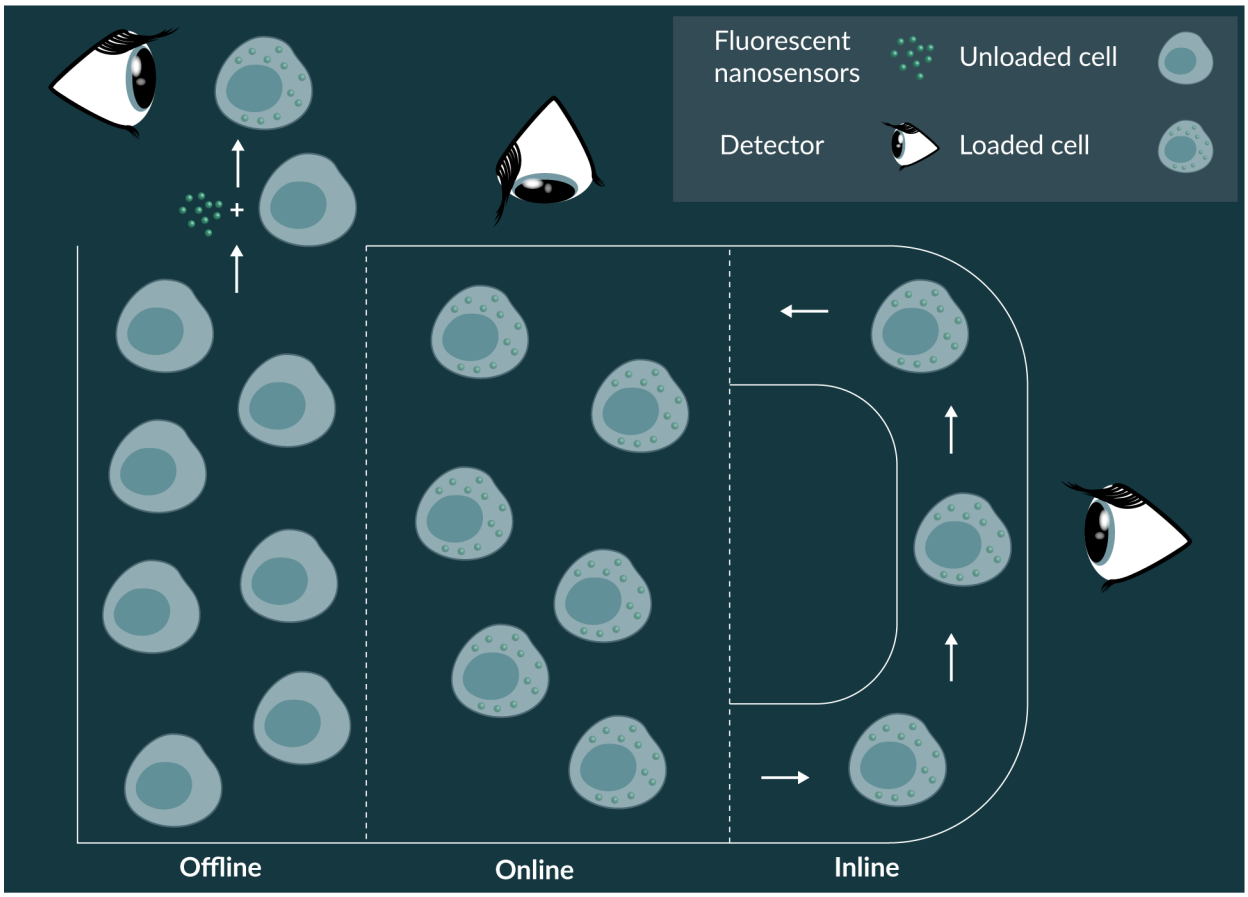
Offline, online & inline analytics

Offline cell culture is a form of indirect measurement, where samples are extracted from the bioreactor and analysed at an independent location [83]. Extraction of sample can permit a greater variety of analytics to be conducted, as samples can be subjected to an array of assays that cannot be readily integrated into the bioreactor. However, when the extraction vessel is different from the bioreactor, especially if the growth conditions are altered and the samples are not stable, interaction with new environments may introduce measurement artefacts. Consequentially, this may hinder utility of newly acquired analytics for optimization growth parameters. Furthermore, extraction away from the bioreactor reduces the potential for real-time feedback and reduces sample size as it cannot be return to the culture vessel.

The automated analytics provided by direct online monitoring permits real-time *in situ* measurement of analytes of interests. This closes the feedback loop, or permits automated analytics, such that growth conditions and parameters can be attenuated to optimise cell growth conditions [84]. Inline monitoring, a direct measurement technique, can be described as a hybrid offline/online measurement systems, where cell culture systems are continually fed and monitored at a location adjacent to or within the bioreactor and can be recirculated into the growth vessel after analysis, preventing loss of sample [85]. Inline systems are useful when bulk cell culture system are turbid, preventing spectroscopic analysis, or possess high fluid flow rates during mixing, which might generate unwanted measurement artefacts. Cell culture parameters are automatically analysed and attenuated to optimise growth. Due to the automated analytics afforded

FIGURE 3

Diagrammatic representation of offline, online and inline measurements permitted with the aid of optical fluorescent nanosensors.



by online and inline monitoring, both cell growth and resource management are optimized.

Implementation of nanosensors for cell therapy

Fluorescent nanosensors can be incorporated in sub cellular spaces or distributed throughout the system to provide feedback of extracellular parameters [86]. Delivery of fluorescent nanosensors to subcellular spaces is a resource efficient method of obtaining cell specific information, especially for research purposes. This is because, although fluorescent nanosensors have demonstrated low toxicity [87], their ultimate use in humans is yet to be determined. Therefore, for mass production of cells for clinical use an alternative measurement system may be required. However, when fluorescent nanosensors are distributed throughout the bioreactor, vast quantities of particles will be required. These particles could be recovered for repeat use, but in reality, this may not be possible as they may introduce biologically active contaminants, which may subsequently alter the viability of batches.

Fluorescent nanosensors could be used for offline, online and inline measurement for cell culture parameters in bioreactors (Figure 3). With respect to offline monitoring, cells

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could be extracted and then treated with nanosensors to determine intra or extracellular biochemical parameters [88]. For bioreactors, intracellular biochemical parameter characterization would consist of a very large time lag as particles would have to be initially delivered and validated to ensure delivery of particles has not induced biochemical changes [89], and therefore may not be valid for automated analytics.

Fluorescent nanosensors are ideally suited to online and inline measurement systems for research purposes and prior to large-scale clinical application. Cell measurements could be conducted by direct online measurement of culture system with fluorescent microscopy or spectroscopy or inline using methods such as integrated fluorescent flow cytometry. Bioreactors that permit visualisation of growth conditions have been developed [90], whereas bioreactors with integrated flow cytometers are emerging for research purposes. From a practical point of view, offline fluorescent nanosensor technologies would need to address an analytical niche currently occupied by powerful established methods, such as flow cytometry, liquid chromatography, and spent media analysis. Therefore, fluorescent nanosensors could be utilized for established techniques, such as flow cytometry, when conventional antibody-based tools are not available or are not able to perform the dynamic biochemical parameter measurements afforded by fluorescent nanosensors.

FUTURE PERSPECTIVES

Biosensors such as fluorescent nanosensors are the future of cell and gene therapy manufacture as they enable online and inline monitoring of the cellular microenvironment. Automated analytics of culture environments permits real-time realignment of ideal biochemical parameters, through data driven allocation of key resources, such as nutrients and growth factors, as well as eliminating waste. It is anticipated the developments in this field provide great promise to further understand the cellular microenvironment and will pave the way forward for cell and gene therapies.

"Science has given to him an acquaintance with the different relations of the parts of the external world; and more than that, it has bestowed upon him powers which may be almost called creative; which have enabled him to modify and change the beings surrounding him, and by his experiments to interrogate nature with power, not simply as a scholar, passive and seeking only to understand her operations, but rather as a master, active with his own instruments"

–Sir Humphry Davy
(1778–1829) [91]

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AFFILIATION

Veeren M Chauhan

School of Pharmacy, University of Nottingham, Boots Science Building, Science Road, NG7 2RD, UK
E-mail: veeren.chauhan@nottingham.ac.uk

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