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Optical Single Cell Resolution Cytotoxicity Biosensor Based on Single Cell Adhesion Dot Arrays

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Abstract

Low cost easy to use cell viability tests are needed in the pharmaceutical, biomaterial and environmental industry to measure adverse cellular effects. We present a new methodology to track cell death with high resolution. Adherent cells commonly detach from the surface when they die. However, some toxic compounds promote adhesion of dead cells. A methodology that enables both, dynamic detachment monitoring, but also rapid detection of toxic effects of compounds that promote cell adhesion would constitute a step forward towards high throughput cytotoxicity measurements. We achieved dynamic digital quantification of cell viability by simple optical imaging using “Single Cell Adhesion Dot Arrays” (SCADA), fibronectin (FN) dot arrays designed to accommodate a single cell on each fibronectin dot. For cytotoxicity measurements, cell-filled SCADA substrates were exposed to K_2CrO_4, $HgSO_4$ salts and dimethyl sulfoxide (DMSO). The toxic effect of DMSO and K_2CrO_4 was dynamically monitored by measuring cell detachment rate during more than 30 hours by quantifying the number of occupied dots in the SCADA array. $HgSO_4$ inhibited cellular detachment from the surface, and cytotoxicity was monitored using Trypan Blue life/death assay directly on the surface. In all cases, the cytotoxicity effects were easily monitored with single cell resolution, and the results were comparable to previous reports. SCADA enabled dynamic measurements at the highest resolution due to the digital measuring in this method. The integration of SCADA substrates into microfluidic platforms, will provide a practical tool that will extent to fundamental research and commercial applications.

Keywords

Biosensor, cell adhesion, optical sensors, cytotoxicity, diagnostics, microtechnology

1. Introduction

Cytotoxicity assays are mostly used for drug screening and testing of chemical induced cellular death. These tests are essential in basic research, in the pharmaceutical industry and in the elaboration of environmental regulation. There are several cytotoxicity tests available, a number of them are based on detecting specific metabolic processes through colorimetric or fluorometric assays, such as crystal violet, MTT, Annexin V, or Trypan Blue among others [1]. Another well-established method is the Colony Formation Assay (CFA) for cytotoxicity testing, which implies the quantification of the ability of cells to form colonies, counting by eye or by microscopy the number of cell colonies created during several days or weeks [1,2]. On the other hand, flow cytometry provides accurate quantitative measurements of cellular death with single cell resolution, and offers the possibility of performing high throughput (HTP) analysis. However, flow cytometry analyses cells in suspension, it cannot be performed on surface, and cellular staining is required, preventing the possibility of real-time monitoring [3]. Real-time cytotoxicity monitoring tests, such as Scalable Time-lapse Analysis of Cell death Kinetics (STACK) technology are also capable of performing high throughput (HTP) analysis of death dynamics similarly to flow cytometry. In these cases, fluorescence is used to identify alive and dead cell populations using fluorescence microscopy and careful optimization of image analysis routines [4]. In addition, a system comprised of a high-density array of micro-cavities for single cells has been reported as a fluorescent-based single cell cytotoxicity assay. In this work, the array of individually entrapped cells enabled the precise quantification of dead cells that had been previously labelled with a fluorescent live/dead staining (5).

In order to develop a simple to use and low-cost test to facilitate the screening of cytotoxicity at the point of need, it would be of interest to have a simple test that could provide cellular

death kinetics with minimal intervention of the user and that do not need highly specialized equipment to read out the test.

Upon exposure to toxic compounds, cells can lose their adhesion capability resulting in cell detachment from a surface. A technology called xCELLigence Real-Time Cell Analysis (RTCA) monitors the effect of a toxic compound on a cell culture, monitoring the detachment rate with a microelectrode-patterned surface. The electrochemical impedance of the electrode based substrate changes in relation to the number of cells adhered to their surface, providing a label-free, real-time cell analysis platform for cell growth and detachment. Even though it is a very sensitive technique to monitor cellular responses to toxics, it is a non-specific technique, because morphological changes in the cells also cause alterations in the impedance values. Therefore, it is not possible to distinguish between cellular detachment and a change in morphology without external labels [5-8]. Additionally, impedance based techniques would not provide the means to distinguish between a living cell or a dead cell that remains attached to the surface because in both cases the impedance signal would be similar.

A methodology that enables both, dynamic detachment monitoring, but also rapid detection of toxic effects of compounds that promote cell adhesion would constitute a step forward towards high throughput cytotoxicity measurements.

Protein patterning techniques enable the creation of cell arrays adhered to surfaces which have been used for many studies in cell biology, morphogenesis, cell polarity, cell division axis [9], whole blood platelet isolation and characterisation [10-12] and high throughput analysis [13]. Recently, we introduced the use of Single Cell Adhesion Dot Array (SCADA) substrates as an optical biosensing platform for accurate quantification of cell affinity for protein substrates with single cell resolution [14].

Herein we report an optical method to monitor cell death with single cell resolution using SCADA substrates, by accurate quantification of cell detachment and/or trypan blue staining **(Figure 1).** This is an application of SCADA substrates that to the best of our knowledge it has not been explored before. The ordered distribution of individually adhered cells on the protein dot array, combined with optical detection, enables digital and dynamic monitoring of cell detachment or individual staining triggered by cell death without the need of fluorescent read out. Such a measurement may have high potential for applicability in cytotoxicity measurements.

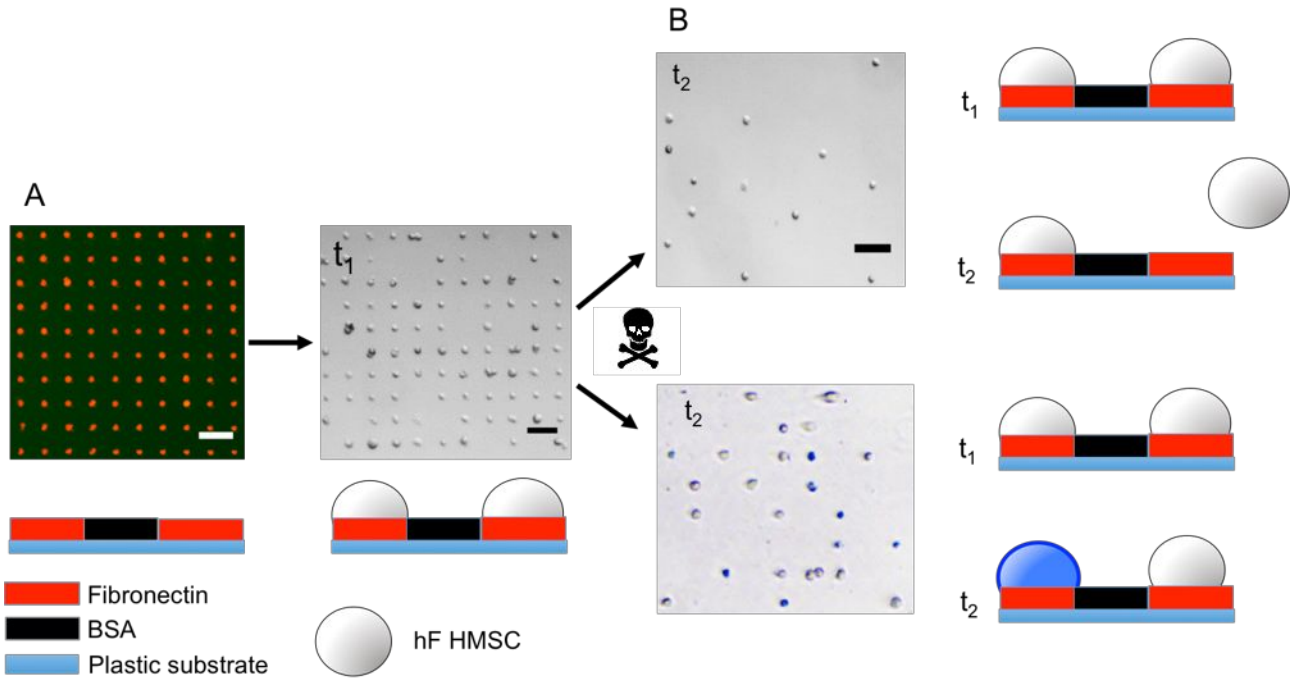

Figure 1. Scheme of Cytotoxicity assay using SCADA substrates. A) Left: Fluorescence microscopy image of a fluorescent labelled FN-SCADA substrate, comprised of an array of FN dots; right: bright field microscopy image of the FN-SCADA substrate after incubation with a cellular suspension, showing single cell adhesion to the FN dots of the SCADA substrate. B) Bright field microscopy images of the cell-saturated SCADA substrates after incubation with two types of toxic compounds that caused cell death and triggered either cell detachment (top) or colour stain (bottom). Scale bar corresponds to 100 μm.

2. Materials and methods

2.1. Materials

2.1.1. Photolithography and Soft Lithography

Dow Corning Sylgard 184 was purchased from Ellsworth Adhesive for the fabrication the Polydimethylsiloxane (PDMS) stamps needed for micro-contact printing. The photo mask for the fabrication of the masters for the PDMS stamps was ordered to JD Photodata. Trimethoxy (octadecyl) silane was purchased from Sigma Aldrich to protect the patterned silicon masters for the peeling off of PDMS. Silicon wafers (1-100 ohm-cm 500 μm) were purchased from University Wafer Inc., and SU-8 2025 photoresist and SU-8 developer were obtained from ATIS S.A. (Spain).

2.1.2. Fabrication of Single Cell Adhesion Dot Arrays (SCADA) by Micro-contact Printing.

For the creation of protein patterns, Bovine Plasma Fibronectin (FN) and Tetramethylrhodamine conjugated Albumin from Bovine Serum (BSA) were purchased from Fisher Scientific (Life Technologies, Spain). Phosphate Buffer Saline tablets from Sigma Aldrich were used to make 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride (pH= 7.4 at 25 ºC). Bovine Serum Albumin was purchased from Sigma Aldrich for the preparation of 1% BSA as blocking solution. Polystyrene (PS) P12 culture dishes from Fisher Scientific Spain were used as substrates. For the incubation of cell suspension in the patterned wells, Vari-Mix steep angle rocker was purchased from Fisher Scientific Spain.

2.1.3. Cell source and materials for cell culturing.

Human adult Mesenchymal Stem Cells were obtained from human hair follicles (hHF MCSs). Growth Medium (GM) consisted of Dulbecco's Modified Eagle's Medium (DMEM)

(Fisher Scientific Spain) supplemented with 30% Fetal Bovine Serum (FBS) (Fisher Scientific Spain) and 10% Penicillin/Streptomycin (P/S) (Fisher Scientific Spain). Medium for incubation on patterning consisted in Dulbecco's Modified Eagle's Medium (DMEM) (Fisher Scientific Spain) with 10% Penicillin/Streptomycin (P/S) (Fisher Scientific Spain). For cell viability quantification, Gibco Trypan Blue Solution (0.4 %) was purchased from Fisher Scientific Spain. Paraformaldehyde 4% for fixation was purchased from Panreac Quimica Spain.

For the performance of the cytotoxicity test, Potassium Chromate (K_2CrO_4) and Mercury Sulfate ($HgSO_4$) were purchased from Merck and from Panreac Quimica Spain, respectively. Dimethyl Sulfoxide (DMSO) was purchased from Fisher Scientific Spain.

2.2. Methods

2.2.1. Fabrication of PDMS stamps. Master fabrication by photolithography.

SU-8 mould fabrication station (BlackHole-Lab) was used for the manufacturing of the SU-8 master on silicon wafers. The silicon wafer was first dehydrated at 220 ºC, and then SU-8 2025 was dynamically spun on top of the wafer, reaching a maximum of 5500 rpm. The wafer was soft baked for 2 minutes at 65 ºC and 4 minutes at 95 ºC. To achieve the adequate resolution of the design of the photo mask, the rigid surface was exposed to UV light during 4 pulses of 5 seconds each, and then it was baked on the hot plate for 1 minute at 65 ºC and 3 minutes at 95 ºC. Finally, the features were developed dipping the master in the developer in 2 cycles of 1 minute. After the fabrication of the master, it was silanised, adding a layer of trimethoxy (octadecyl) silane by vapour deposition for 1 hour, to avoid damages to the master when releasing the PDMS. PMDS stamps were made by pouring a mixture of Sylgard 184 elastomer and curing agent (10:1 v/v) over the fabricated silicon master. It was degassed and cured at 60 ºC for 1 h, then the PDMS was detached from the wafer and it

was kept at 60ºC for 16 hours more to make sure the crosslinking was completed. To get the stamps, the PDMS mould obtained was cut in pieces of 1 cm x 1 cm. Each stamp was comprised of 20 000 pillars of 20 µm diameter with a separation of 50 µm between dots.

2.2.2. Surface patterning by Micro-contact Printing.

The wells of PS P12 culture dishes were used as substrates for protein patterning and the process was performed at explained elsewhere [15]. Briefly, first the PDMS stamps were inked with a solution of 115 nM of fibronectin and 100 nM of rhodamine labelled BSA (BSA-TAMRA) in PBS for 30 minutes. During that incubation time, the polymeric surfaces of the culture dishes were oxidised with air plasma for 40 seconds, to make the surface more hydrophilic and to enhance the protein transfer from the PDMS stamp to the substrate. The excess of protein ink solution was removed with the micropipette, the PDMS stamps were rinsed with distilled water, and they were then carefully dried with compressed air to eliminate humidity. The patterned area of the stamps was put in contact with the substrate for 30 minutes, and then the stamps were removed, creating a protein pattern on the surface, comprised of 20.000 dots of 20 µm diameter and separated from each other by 50 µm. Finally, 1 mL of 1% BSA solution in PBS was added to each patterned well to block any surface area that had not been in contact with proteins. The blocking solution was kept overnight at 4 ºC. For every experiment, the surface patterning for cell adhesion was carried out the day before the addition of the cells was performed.

All the substrates were characterized by fluorescence microscopy to evaluate the quality of the FN dot array. We analysed the fluorescence intensity and the diameter of 3.300 dots within a single substrate, and we considered a substrate as adequate if the coefficients of variation (CVs) in their fluorescence intensity and their shape were lower than 20%. See supporting information for a fluorescence microscopy image of the FN dot array.

2.2.3. Quantification of cell adhesion and detachment.

hHF-MSCs cultured in T75 PS flasks were trypsinizsed, and after centrifugation cells were quantified to obtain an adequate number of cells. After centrifugation, they were resuspended in pattern medium, comprised by DMEM and P/S (9:1 v/v) in a concentration of 100.000 cell·mL^{-1}. 1 mL of cell suspension was added to each patterned well, which was previously washed 3 times with PBS, and incubated at 37ºC and 5% CO_2 on a rocker (steep angle rocking), at a speed of 5 cycles per minute for at least 90 minutes, in order to achieve a Dot Array Occupancy (DAO) over 90%. After that, the wells were washed twice with PBS, and subsequently PBS or a solution of a toxic compound was added to the medium. Cellular adhesion was monitored for several hours, keeping cells at 37 ºC and 5% of CO_2. To quantify the number of cells adhered on a patterned surface, bright field microscopy images were taken. DAO was calculated as the percentage of binding sites (fluorescent dots) occupied by cells. Cellular detachment was calculated by subtracting the DAO value at a certain time from the initial DAO normalized to 100 %.

The homogeneity of the adhesion/detachment was evaluated along the whole substrate, containing 20.000 dots. The average CV among the DAO calculated along the whole substrate using 3 random images containing 1.728 spots was 4.71% (see supporting information for raw data images). Each data point in the manuscript corresponds to the arithmetic average of DAO among 3 different replicas of the same sample type. For each replica, 1 random image was taken, with a field of view of 13.5 mm^2, each replica comprised of 1.728 protein dots. Error bars correspond to the standard deviation among the three different replicas.

Trypan Blue test was performed in other samples to confirm that the adhered cells were alive, cell media was removed, the samples were washed twice with PBS, and 50% (v/v) of Trypan Blue in DMEM was added to immediately track their viability. Optical microscopy

pictures were taken and cellular viability was measured by counting the number of blue stained cells and divided by the total number of cells.

2.2.4. Cellular viability of PBS exposed samples by flow cytometry.

To calibrate the system, 1200 µL of trypsinizsed cells were collected from a standard culture flask, and 250 µL of ethanol (70%) and 10 µL of ethidium bromide were added to cause and detect cell death, respectively. Subsequently, cells were exposed to PBS lacking Ca^{2+} and Mg^{2+}, and cell adhesion data was collected at different time points to determine rate of live and death cells on the detached cells. 10 µL of ethidium bromide were added per 1200 µL of detached cells containing media. Those samples were introduced in the cytometer with the previously determined settings for those cells, and the percentage of dead cells was calculated. Ratios alive/dead cells of 63/37, 72/28, 60/40 was obtained after 1, 2 and 3h incubation of the SCADA substrate with PBS.

2.2.5. Microscopy images.

A Nikon Eclipse TE2000-S inverted microscope coupled with ANDOR ZYLA sCMOS and C-LHG1 100W Mercury lamp was used to image the fluorescent protein patterns and bright field images of adherent cells. An Olympus IMT-2 inverted microscope, coupled with a TUCSON BCA 5.0 colour camera was used to perform the trypan blue viability assay on the SCADA substrates.

3. Results

3.1. *Monitoring cell adhesion and detachment to protein substrates with single cell resolution.*

Fibronectin (FN) is a protein of high molecular weight from the Extracellular Matrix (ECM), which plays a crucial role in cell adhesion, as it binds to integrins located in the cell membrane. On the other hand, primary human hair follicle Mesenchymal Stem Cells (hHf-MSCs), are adherent cells that express a number of integrin molecules like α1, α2, α3, α4, α5, αv, β1, β3 and β4, some of which have been previously reported to be involved in MSC adhesion [16]. In order to demonstrate the suitability of micro-patterned SCADA substrates to monitor cellular death, first the adhesion and detachment kinetics of hHf-MSCs to a FN-SCADA substrate were monitored. Arrays of 20 μm FN dots with inter dot space of 50 μm were created on polystyrene (PS) cellular culture dishes by micro-contact printing, and the remaining surface of the dish was blocked with Bovine Serum Albumin (BSA) to avoid non-specific cellular adhesion. To evaluate the homogeneity of the composition of the protein dot array, the fluorescence intensity and the diameter of 3.300 dots were analysed within a single substrate. The coefficient of variation (CV) for fluorescence intensity and dot diameter values were of 6 % and 14 % respectively, confirming the homogeneity of the dot composition and their shape along the substrate. 1 mL of 100.000 cell·mL^{-1} suspension was added to each patterned well, and kept incubating at 5 cycles per minute (steep angle rocking). To determine adhesion kinetics, the occupancy of the dot array (DAO) was measured after 15', 30', 45', 1 h, 1.5 h, 2 h, 2.5 h, 3 h and 3.5 h, taking optical microscopy images of the substrates at each time point, and calculating the ratio of occupied binding dots. As shown in Figure 2, cells started to attach to FN dots in a few minutes, and just after 30 minutes of incubation, there was more than 50% occupancy of the protein dot array. After 2 h of incubation, hHF-MSCs reached the adhesion plateau with a DAO of 97%. Controlled cellular detachment was induced by exposing the cell-saturated substrates to a solution of PBS lacking Ca^{2+} and Mg^{2+}. Integrins have 3 to 5 binding sites for divalent cations in each heterodimer. Different cations play different roles; they may act as adhesion promoters

inducing ligand binding, but also as antagonists inhibiting adhesion [17]. It is known that the absence of Ca^{2+} and Mg^{2+} in the media affects the equilibrium between the active and the inactive forms of integrins, and promotes cellular detachment without damaging the cells [18]. hHF-MSCs detached from FN dots slowly in the first 40 minutes, whereas from that time on, their detachment kinetics increased significantly. After 2 hours in PBS, cellular release from the surface became slower, until every adhered cell was detached, after 4 hours (Figure 3). Flow cytometry confirmed that more than 60 % of detached cells remained alive, confirming the controlled detachment triggered by the lack of Ca^{2+} and Mg^{2+}. This experiment proved the capability of this technique to perform a label-free, quantitative and dynamic monitoring of cellular detachment with single cell resolution by using optical microscopy.

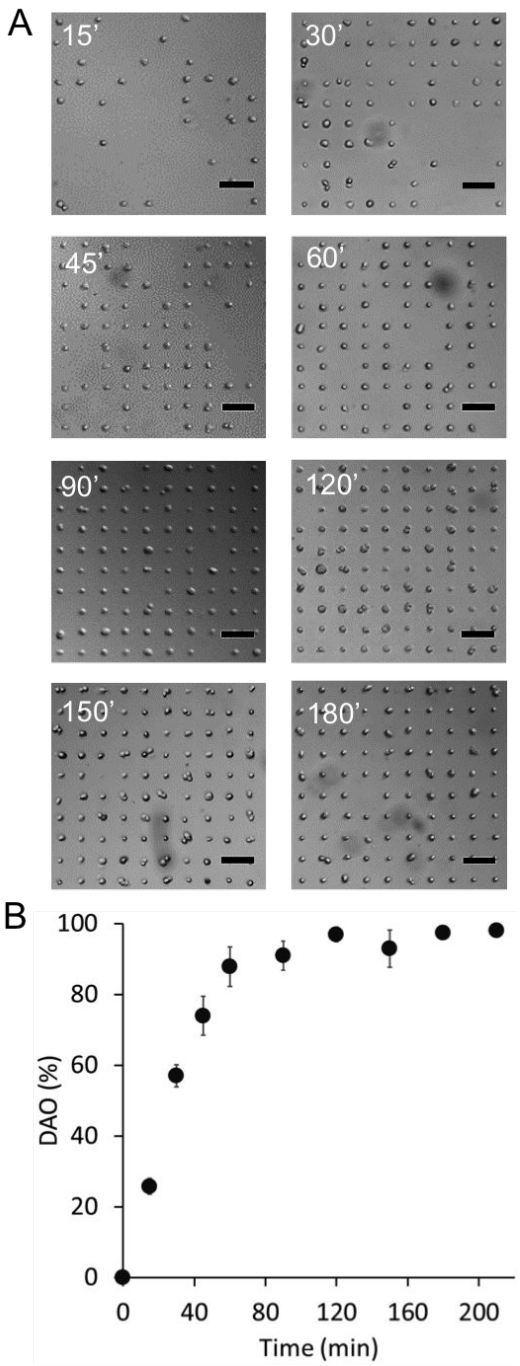

Figure 2. Dynamic cell attachment on SCADA. A) Optical microscopy, bright field, images of single cell adhesion to the dot array at different time points: (i) 15 min, (ii) 30 min, (iii) 45 min, (iv) 1 h, (v) 1.5 h, (vi) 2 h, (vii) 3 h and (viii) 3.5 h. B) Dot Array Occupancy (DAO) versus time. Scale bar corresponds to 100 μm. Error bars correspond to the standard deviations (n=3)

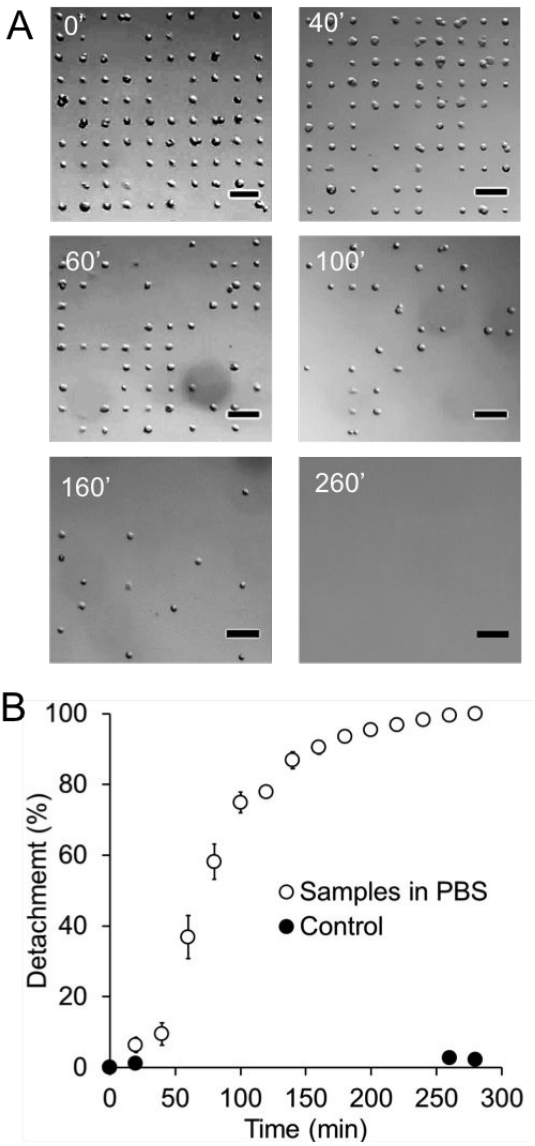

Figure 3. Dynamic cell detachment on SCADA. A) Optical microscopy images of single cell arrays at different times in presence of PBS: (i) at time 0, (ii) 40 min, (iii) 60 min, (iv) 100 min, (v) 160 min and (vi) 260 min. Scale bar corresponds to 100 μm. B) Detachment of hHF-MSCs versus time of exposure to PBS. Error bars correspond to the standard deviations (n=3)

3.2. Monitoring cytoxicity of K_2CrO_4 and DMSO measured by label free SCADA viability test

Potassium chromate is a strong oxidant agent, which is considered to be highly toxic, while DMSO is an organosulfur compound widely used for biological applications. DMSO induces membrane permeabilisation in living cells that ends up compromising membrane integrity for higher concentrations than 10% (v/v), for this reason it is usually used at lower concentrations for cell culture [19].

To monitor K_2CrO_4 cytotoxicity, cell saturated SCADA substrates were incubated with solutions of 50 μM and 100 μM of K_2CrO_4 for 30 hours. The data obtained showed increasing cellular detachment with the exposure time and the concentration of the toxic, after six hours of incubation **(Figure 4)**. After 30 hours in presence of 50 and 100 μM of K_2CrO_4, there was a 74 % and 87 % of cellular detachment in contrast with the control samples which had only a 30 % of detachment. Cr (VI) has been previously reported to cause replication stress [20], which can lead to apoptosis or programmed cellular death [21,22]. This agrees with the results showed in this paper, significant detachment could only be observed after 6 hours of incubation with the toxic.

To evaluate the cytotoxicity of DMSO, the effect of, 1, 2, 6, 8 and 10% (v/v) DMSO was measured on cells adhered to FN-SCADA substrates for 24 h. Our measurements showed an increasing detachment of cells with time of exposure to DMSO, reaching up to 40 % detachment after 5 hours for the highest concentration of DMSO in contrast with the control samples which were not exposed to DMSO and exhibited only a 4% detachment at the same time. When cells were treated with 8% and 10% of DMSO (v/v), after 21 hours 100% of the cells were detached from the substrate, while in the control sample only a 16% of detachment was observed. In general, faster detachment kinetics was observed for higher concentrations of DMSO **(Figure 4)**. To the best of our knowledge, the effect of DMSO

concentrations below 10% for hHF MSCs has not been precisely quantified before in terms of cellular death.

When the cytotoxicity of K_2CrO_4 and DMSO were evaluated, trypan blue viability test showed that all the cells that remained adhered to the substrate were alive, while the cells detached were dead. These tests revealed a good correlation between cellular death and cell detachment from the SCADA substrates.

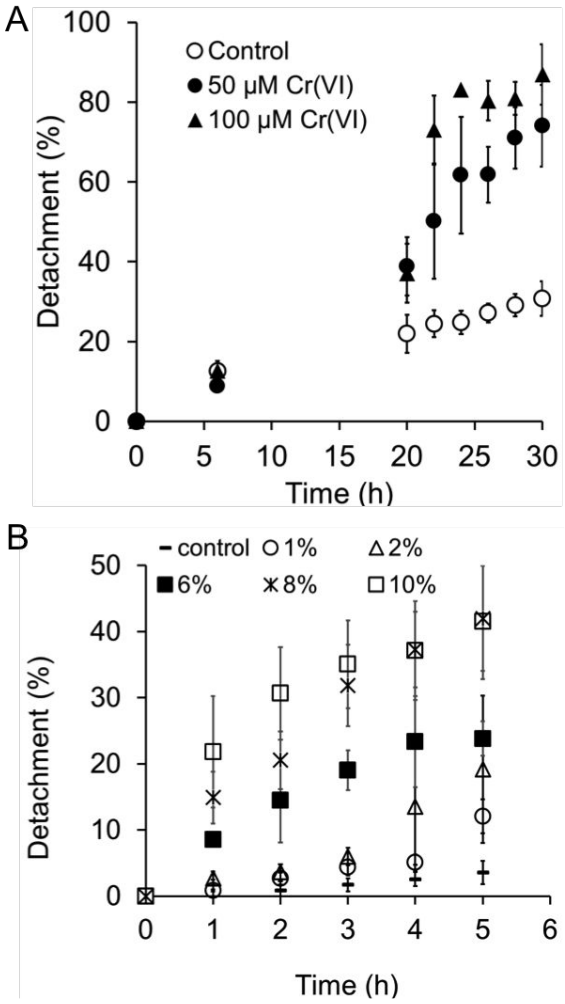

Figure 4. Cellular detachment kinetics in presence of 50 and 100 µM K_2CrO_4 for 30 hours (A) and 1, 2, 4, 6, 8 and 10 % of DMSO for 5 hours (B). Error bars correspond to the standard deviations (n=3)

3.3. Cytotoxicity colorimetric SCADA viability test

In contrast to impedance-based techniques, the SCADA substrates could also be used to detect the toxic effect of compounds that promote stable cell adhesion after cell death. This is thanks to the fact that the SCADA methodology uses an optical read out.

Hg (II) is a divalent cation promotes stable adhesion of hHF-MSCs on FN. In this work, this model was used to monitor cell death by a colorimetric assay using the binary counting board of occupied/vacant. Cell containing SCADA substrates were exposed to $HgSO_4$ solutions of 5, 25 and 50 μM for 30 hours. In the presence of $HgSO_4$ cells did not detach from the fibronectin surface, the DAO values were similar to the DAO values of the control samples (**Figure 5A**). To monitor cell death in this case, trypan blue was used, a life/death marker that penetrates the cellular membrane of dead cells, staining them in blue. As it cannot enter through healthy cellular membranes, living cells remain uncoloured (**Figure 5B**). Subsequently, cell saturated FN-SCADA substrates were exposed to 50 μM of $HgSO_4$ for 48 hours, and trypan blue was added at different time points to measure cell viability. The quantification of blue and non-coloured cells on the substrate at each time point showed an increasing number of dead cells with the time of exposure to the toxic compound **(Figure 5C)**. In a different experiment, cells adhered to FN-SCADA substrates were exposed to solutions of 5 μM, 25 μM and 50 μM of $HgSO_4$ for 48 hours, and the trypan blue assay also showed an increasing rate of cell death with the concentration of $HgSO_4$, confirming its cytotoxicity (**Figure 5D**).

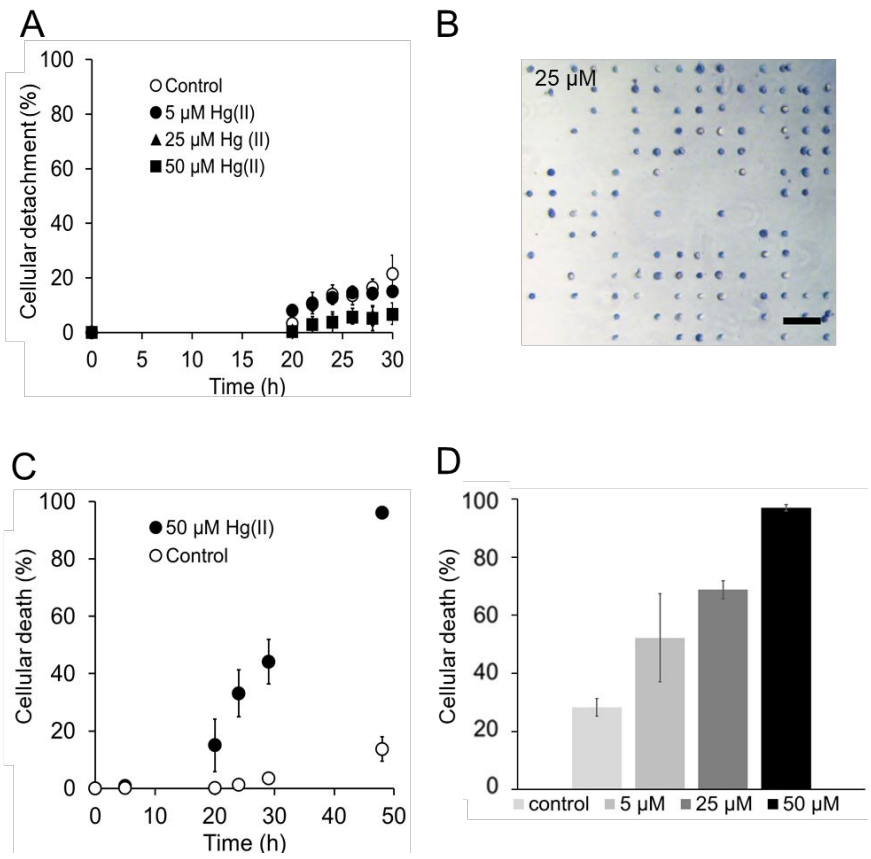

Figure 5. Cellular death after incubation with Hg (II). A) Cellular adhesion kinetics of hHF MSCs in presence of 5, 25 and 50 µM of $HgSO_4$. B) Optical microscopy image (10 x objective) of FN-SCADA substrates with hHF MSCs 48 h after incubation with 25 µM of $HgSO_4$. Scale bar corresponds to 100 µm. C) Graphical representation of cellular death during 48 hours in presence of 50 µM of $HgSO_4$. D) Plot of cell death after 48 hours in presence of 5, 25 and 50 µM of $HgSO_4$. Error bars correspond to the standard deviations (n=3)

4. Discussion and conclusions

SCADA substrates enabled, for the first time, to measure the toxic effect of DMSO and K_2CrO_4 on hHF MSCs by monitoring cell detachment rate in a label-free mode by optical imaging. The precision of the SCADA system is based on a binary counting mode, which uses an array of adhesive dots occupied by individual cells for the quantification of cellular

detachment with single cell resolution. The number of positive dots can be easily counted, and its percentage from the total number of adhesion dots calculated, being that the dot array occupancy (DAO) or the cellular adhesion percentage. Each adhesion site is independent from each other, having each dot the same probability to be occupied by a single cell, emptied or labelled, constituting a binary digital code: occupied/vacant, stained/not stained, positive/negative or what is the same 1/0.

There are a number of cytotoxicity tests currently available in the market, each of them with different characteristics, and each one providing different type of data. What cytotoxicity test to use may depend on the type of cell and the type of data required by the user. Herein, we include a decision tree to help the user decide whether a SCADA test could provide the information needed for their specific system (**Figure 6**): SCADA cytotoxicity test is a non-invasive, specific technique useful to obtain cellular death kinetics with single cell resolution, using simply optical instrumentation. SCADA is a highly sensitive technique, designed to monitor cell death. Toxic effects which do not result in cell death cannot be detected with this platform. This methodology enables both, dynamic detachment monitoring, and also rapid detection of toxic effects of compounds that promote cell adhesion.

PBS induced cellular detachment through integrin deactivation, lowering down the capability of cells to attach to fibronectin, which resulted in a 100% detachment rate in 5 hours. While Cr (VI) induced cellular detachment by triggering the apoptosis of cells, which is a much slower process. DMSO triggered cell detachment inducing cellular death, while Hg (II) killed the cells as confirmed by Trypan Blue test, but kept cells strongly adhered to fibronectin dots. This divalent cation, could be modulating cellular adhesion activating or inactivating integrins through specific divalent cation-binding sites.

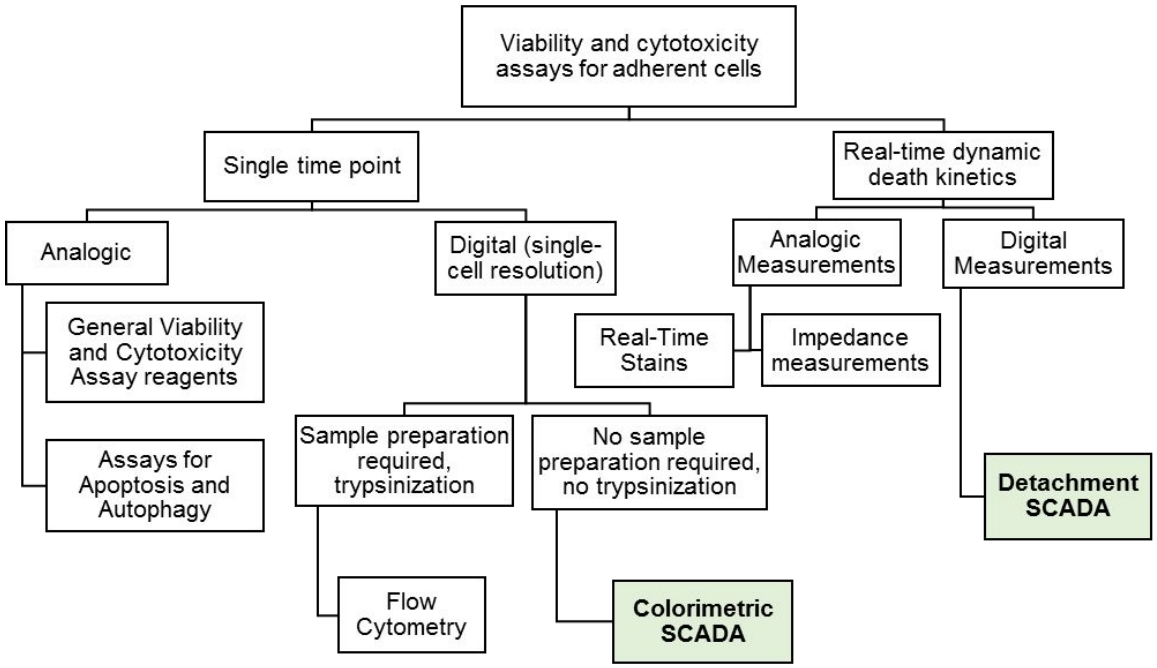

Figure 6. Classification of viability and cytotoxicity assays already available for adherent cells as a function of the type of data that may be obtained. SCADA provides digital measurements of cellular death without the need of sample preparation after exposure to the toxic.

In contrast with current available cytotoxicity tests based on analogical signals like fluorescence, light or electrochemical impedance, SCADA is based on digital counting, providing the highest resolution, up to single cell resolution. Besides, compared to flow cytometry, SCADA methodology allows dynamic and real time measurements, because it avoids the time gap between the target time when the toxic is added and the time in which the cellular death measurements are performed. SCADA assays require only a patterned substrate area of less than 1 cm^2, and a reduced number of cells, ranging from hundreds to thousands of cells, while other techniques normally use more than 10.000 cells per measurement. Nonetheless, the number of cells analysed in SCADA could be increased to a million cells if needed, by using larger patterned areas of the substrate and automated

microscopy scanning. For this proof of concept, the patterned substrates were created by micro-contact printing, but other methods such as light directed chemical patterning could be used by any laboratory with commercially available equipment.

A combination of assays such as colorimetric or flow cytometry may be necessary to correlate viability and cellular adhesion in the presence of a specific compound to test. Once determined that correlation, the system can provide extra information such as toxicity kinetics or cell population heterogeneity. The readout of this assay requires only optical components to be carried out, avoiding the need of high sensitivity detectors like in the case of fluorescence measurement, or complex fabrication process of microelectrode arrays used in the xCELLigence RTCA. The applicability of SCADA cytotoxicity methodology could be extended to many different types of cells and assays, by customising the protein dot array, changing the shape of the dots, their number and their composition. We believe that the impact of this methodology for accurate measurement of cell cytotoxicity will extent to fundamental research and commercial applications through the integration of SCADA substrates into microfluidic devices with low cost portable optical components, enabling high throughput cytotoxicity measurements at the point of need.

Supporting Information: Homogeneity evaluation of protein patterns. Quantification of cell adhesion and detachment over 20.000 dots. Cell death kinetic analysis with 1.000 cells.

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