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1 # Low Cost Single Cell Resolution Cytotoxicity Biosensor Based on

2 # Single Cell Adhesion Dot Arrays

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25 ## Abstract

Low cost easy to use cell viability tests are needed in the pharmaceutical, biomaterial and environmental industry to measure adverse cellular effects. Herein we present a new methodology to track cell death with high resolution. We achieved dynamic digital quantification of cell viability by simple optical imaging using “Single Cell Adhesion Dot Arrays” (SCADA). Fibronectin (FN) dot arrays were fabricated on cell culture multiwell plates. The dot array was designed to accomodate 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). Adherent cells commonly detach from the surface when they die. Dynamic monitoring of the toxic effect of DMSO and K_2CrO_4 was done 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. Cytotoxicity SCADA tests require only a transparent substrate, with a patterned area of less than 1 mm^2 and a reduced number of cells. SCADA enabled dynamic measurements at the highest resolution due to the digital measuring of this methodology. Integrated into microfluidic platforms, SCADA will provide a practical tool that will extent to fundamental research and commercial applications.

Keywords

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

47 ## 1. Introduction

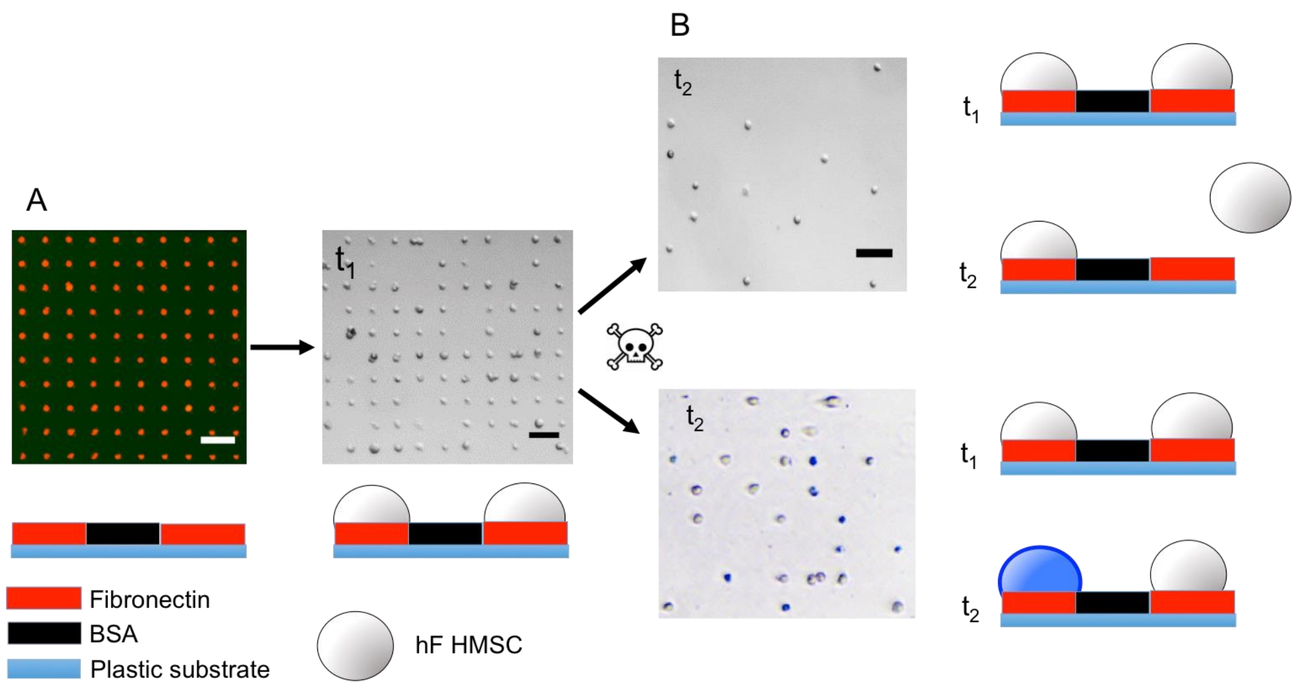
48 Cytotoxicity assays are mostly used for drug screening and testing of chemical induced
49 cellular death. These tests are essential in basic research, in the pharmaceutical industry
50 and in the elaboration of environmental regulation. There are several cytotoxicity tests
51 available, a number of them are based on detecting specific metabolic processes through
52 colorimetric or fluorometric assays, such as crystal violet, MTT, Annexin V, or Trypan Blue
53 among others (1). Another well-established method is the Colony Formation Assay (CFA) for
54 cytotoxicity testing, which implies the quantification of the ability of cells to form colonies,
55 counting by eye or by microscopy the number of cell colonies created during several days
56 or weeks (1,2). On the other hand, flow cytometry provides accurate quantitative
57 measurements of cellular death with single cell resolution, and offers the possibility of
58 performing high throughput (HTP) analysis. However, flow cytometry analyses cells in
59 suspension, it cannot be performed on surface, and cellular staining is required, preventing
60 the possibility of real-time monitoring (3). Real-time cytotoxicity monitoring tests, such as
61 Scalable Time-lapse Analysis of Cell death Kinetics (STACK) technology are also capable
62 of performing high throughput (HTP) analysis of death dynamics similarly to flow cytometry.
63 In these cases, fluorescence is used to identify alive and dead cell populations providing an
64 analogical read out in contrast to flow cytometry's digital quantification and single cell
65 resolution (4).

66 Upon exposure to toxic compounds, cells suffer disturbances in their membranes and can
67 lose their adhesion capability resulting in cell detachment from a surface. A technology
68 called xCELLigence Real-Time Cell Analysis (RTCA) monitors the effect of a toxic
69 compound on a cell culture using a microelectrode-patterned surface. The electrochemical
70 impedance of the substrate changes in relation to the number of cells adhered to the surface,
71 providing a label-free, real-time cell analysis platform for cell growth and detachment. Even

72 though it is a very sensitive technique to monitor cellular responses to toxics, it is a non-
73 specific technique, because morphological changes in the cells also cause alterations in the
74 impedance values. Therefore, it is not possible to distinguish between cellular detachment
75 and a change in morphology (5-8), neither can it be distinguished between a living cell or a
76 dead cell that remains attached to the surface.

77 Protein patterning techniques enable the creation of cell arrays on surfaces which have been
78 used for many studies in cell biology, morphogenesis, cell polarity, cell division axis (9), whole
79 blood platelet isolation and characterisation (10-12) and high throughput analysis (13).
80 Recently, we introduced the use of Single Cell Adhesion Dot Array (SCADA) substrates as
81 an optical biosensing platform for accurate quantification of cell affinity for protein substrates
82 (14). Moreover, a system comprised of a high density array of micro-cavities for single cells
83 was reported as a fluorescent based single cell cytotoxicity assay (15). The array of
84 individually entrapped cells enabled the precise quantification of dead cells that had been
85 previously labelled with a fluorescent live/dead staining.

86 The ordered distribution of individually adhered cells on the protein dot matrix, combined
87 with optical detection, would enable digital and dynamic monitoring of cell detachment or
88 individual staining triggered by cell death. Herein we report an optical method to monitor cell
89 death with single cell resolution using SCADA substrates, by accurate quantification of cell
90 detachment and/or trypan blue staining **(Figure 1).** This is an application of SCADA
91 substrates that to the best of our knowledge it has not been explored before despite of its
92 high potential for applicability in cytotoxicity measurements.



93

94 **Figure 1.** Schematic representation of the methodology of the cytotoxicity assay on SCADA
95 substrates. Left: fluorescence microscopy image of a fluorescent labelled FN-SCADA
96 substrate, comprised of an array of FN dots; right: brightfield microscopy image of the FN-
97 SCADA substrate after incubation with a cellular suspension, showing single cell adhesion
98 to the FN dots of the SCADA substrate. B) Brightfield microscopy images of the cell-
99 saturated SCADA substrates after incubation with two types of toxic compounds that caused
100 cell death and triggered either cell detachment (top) or colour stain (bottom). Scale bar
101 corresponds to 100 µm.

102

103 2. Materials and methods

104 *2.1. Materials*

105 *2.1.1. Photolithography and Soft Lithography*

106 Dow Corning Sylgard 184 was purchased from Ellsworth Adhesive for the fabrication the
107 Polydimethylsiloxane (PDMS) stamps needed for micro-contact printing. The photomask for
108 the fabrication of the masters for the PDMS stamps was ordered to JD Photodata.

109 Trimethoxy(octadecyl)silane was purchased from Sigma Aldrich to protect the patterned
110 silicon masters for the peeling off of PDMS. Silicon wafers (1-100 ohm-cm 500 μm) were
111 purchased from University Wafer Inc., and SU-8 2025 photoresist and SU-8 developer were
112 obtained from ATIS S.A. (Spain).

113 *2.1.2. Fabrication of Single Cell Adhesion Dot Arrays (SCADA) by Micro-contact Printing.*
114 For the creation of protein patterns, Bovine Plasma Fibronectin (FN) and
115 Tetramethylrhodamine conjugated Albumin from Bovine Serum (BSA) were purchased from
116 Fisher Scientific (Life Technologies, Spain). Phosphate Buffer Saline tablets from Sigma
117 Aldrich were used to make 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137
118 M sodium chloride (pH= 7.4 at 25 ºC). Bovine Serum Albumin was purchased from Sigma
119 Aldrich for the preparation of 1% BSA as blocking solution. Polystyrene (PS) P12 culture
120 dishes from Fisher Scientific Spain were used as substrates. For the incubation of cell
121 suspension in the patterned wells, Vari-Mix steep angle rocker was purchased from Fisher
122 Scientific Spain.

123 *2.1.3. Cell source and materials for cell culturing.*
124 Human adult Mesenchymal Stem Cells were obtained from human hair follicles (hHF
125 MCSs). Growth Medium (GM) consisted of Dulbecco's Modified Eagle's Medium (DMEM)
126 (Fisher Scientific Spain) supplemented with 30% Fetal Bovine Serum (FBS) (Fisher
127 Scientific Spain) and 10% Penicillin/Streptomycin (P/S) (Fisher Scientific Spain). Medium
128 for incubation on patterning consisted in Dulbecco's Modified Eagle's Medium (DMEM)
129 (Fisher Scientific Spain) with 10% Penicillin/Streptomycin (P/S) (Fisher Scientific Spain). For
130 cell viability quantification, Gibco Trypan Blue Solution (0.4 %) was purchased from Fisher
131 Scientific Spain. Paraformaldehyde 4% for fixation was purchased from Panreac Quimica
132 Spain.

133 For the performance of the cytotoxicity test, Potassium Chromate (K_2CrO_4) and Mercury
134 Sulfate ($HgSO_4$) were purchased from Merck and from Panreac Quimica Spain,
135 respectively. Dimethyl Sulfoxide (DMSO) was purchased from Fisher Scientific Spain.
136
137 *2.2. Methods*
138 *2.2.1. Fabrication of PDMS stamps. Master fabrication by photolithography.*
139 SU-8 mould fabrication station (BlackHole-Lab) was used for the manufacturing of the SU-
140 8 master on silicon wafers. The silicon wafer was first dehydrated at 220 ºC, and then SU-8
141 2025 was dynamically spun on top of the wafer, reaching a maximum of 5500 rpm. The
142 wafer was soft baked for 2 minutes at 65 ºC and 4 minutes at 95 ºC. To achieve the adequate
143 resolution of the design of the photomask, the rigid surface was exposed to UV light during
144 4 pulses of 5 seconds each, and then it was baked on the hot plate for 1 minute at 65 ºC
145 and 3 minutes at 95 ºC. Finally, the features were developed dipping the master in the
146 developer in 2 cycles of 1 minute. After the fabrication of the master, it was silanised, adding
147 a layer of trimethoxy(octadecyl)silane by vapour deposition for 1 hour, to avoid damages to
148 the master when releasing the PDMS. PMDS stamps were made by pouring a mixture of
149 Sylgard 184 elastomer and curing agent (10:1 v/v) over the fabricated silicon master. It was
150 degassed and cured at 60 ºC for 1 h, then the PDMS was detached from the wafer and it
151 was kept at 60ºC for 16 hours more to make sure the crosslinking was completed. To get
152 the stamps, the PDMS mould obtained was cut in pieces of 1 cm x 1 cm. Each stamp was
153 comprised of 20 000 pillars of 20 µm diameter with a separation of 50 µm between dots.
154
155 *2.2.2. Surface patterning by Micro-contact Printing.*
156 The wells of PS P12 culture dishes were used as substrates for protein patterning and the
157 process was performed at explained elsewhere (16). Briefly, first the PDMS stamps were

158 inked with a solution of 230 nM of fibronectin and 100 nM of rhodamine labelled BSA (BSA-
159 TAMRA) in PBS for 30 minutes. During that incubation time, the polymeric surfaces of the
160 culture dishes were oxidised with air plasma for 40 seconds, to make the surface more
161 hydrophilic and to enhance the protein transfer from the PDMS stamp to the substrate. The
162 excess of protein ink solution was removed with the micropipette, the PDMS stamps were
163 rinsed with distilled water, and they were then carefully dried with compressed air to
164 eliminate humidity. The patterned area of the stamps was put in contact with the substrate
165 for 30 minutes, and then the stamps were removed, creating a protein pattern on the surface,
166 comprised of 20 000 dots of 20 μm diameter and separated from each other by 50 μm.
167 Finally, 1 mL of BSA solution in PBS (150 μM) was added to each patterned well to block
168 any surface area that had not been in contact with proteins. The blocking solution was kept
169 overnight at 4 ^{o}C. For every experiment, the surface patterning for cell adhesion was carried
170 out the day before the addition of the cells was performed.

171 All the substrates were characterized by fluorescence microscopy to evaluate the quality of
172 the FN dot array. We analysed the fluorescence intensity and the diameter of 3300 dots
173 within a single substrate, and we considered a substrate as adequate if the coefficients of
174 variation (CVs) in their fluorescence intensity and their shape were lower than 20%. See
175 supporting information for a fluorescence microscopy image of the FN dot array.

176 *2.2.3. Quantification of cell adhesion and detachment.*

177 hHF-MSCs cultured in T75 PS flasks were trypsinizsed, and after centrifugation cells were
178 quantified to obtain an adequate amount of cells. After centrifugation, they were
179 resuspended in pattern medium, comprised by DMEM and P/S (9:1 v/v) in a concentration
180 of 100.000 cell/mL. 1 mL of cell suspension was added to each patterned well, which was
181 previously washed 3 times with PBS, and incubated at 37°C and 5% CO_2 on a rocker (steep
182 angle rocking), at a speed of 5 cycles per minute for at least 90 minutes, in order to achieve

183 a Dot Array Occupancy (DAO) over 90%. After that, the wells were washed twice with PBS,
184 and subsequently PBS or a solution of a toxic compound was added to the medium. Cellular
185 adhesion was monitored for several hours, keeping cells at 37 ºC and 5% of CO_2. To quantify
186 the number of cells adhered on a patterned surface, brightfield microscopy images were
187 taken with a 4x objective, showing an area of 1.012 protein dots (22 x 46) per picture. DAO
188 was calculated as the percentage of binding sites (fluorescent dots) occupied by cells.
189 Cellular detachment was calculated by subtracting the DAO value at a certain time from the
190 initial DAO normalized to 100 %.

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

199 *Trypan Blue test* was performed in other samples to confirm that the adhered cells were
200 alive, cell media was removed, the samples were washed twice with PBS, and 50% (v/v) of
201 Trypan Blue in DMEM was added to immediately track their viability. Optical microscopy
202 pictures were taken and cellular viability was measured by counting the number of blue
203 stained cells and divided by the total number of cells.

204

205 *2.2.4. Cellular viability of PBS exposed samples by flow cytometry.*

206 To calibrate the system, 1200 µL of trypsinizsed cells were collected from a standard culture
207 flask, and 250 µL of ethanol (70%) and 10 µL of ethidium bromide were added to cause and

208 detect cell death, respectively. Subsequently, cells were exposed to PBS lacking Ca^{2+} and
209 Mg^{2+}, and cell adhesion data was collected at different time points to determine rate of live
210 and death cells on the detached cells. 10 µL of ethidium bromide were added per 1200 µL
211 of detached cells containing media. Those samples were introduced in the cytometer with
212 the previously determined settings for those cells, and the percentage of dead cells was
213 calculated. A ratio 63/37, 72/28, 60/40 alive/dead cells was obtained after 1, 2 and 3h
214 incubation of the SCADA substrate with PBS.

215

216 *2.2.5. Microscopy images.*

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

222

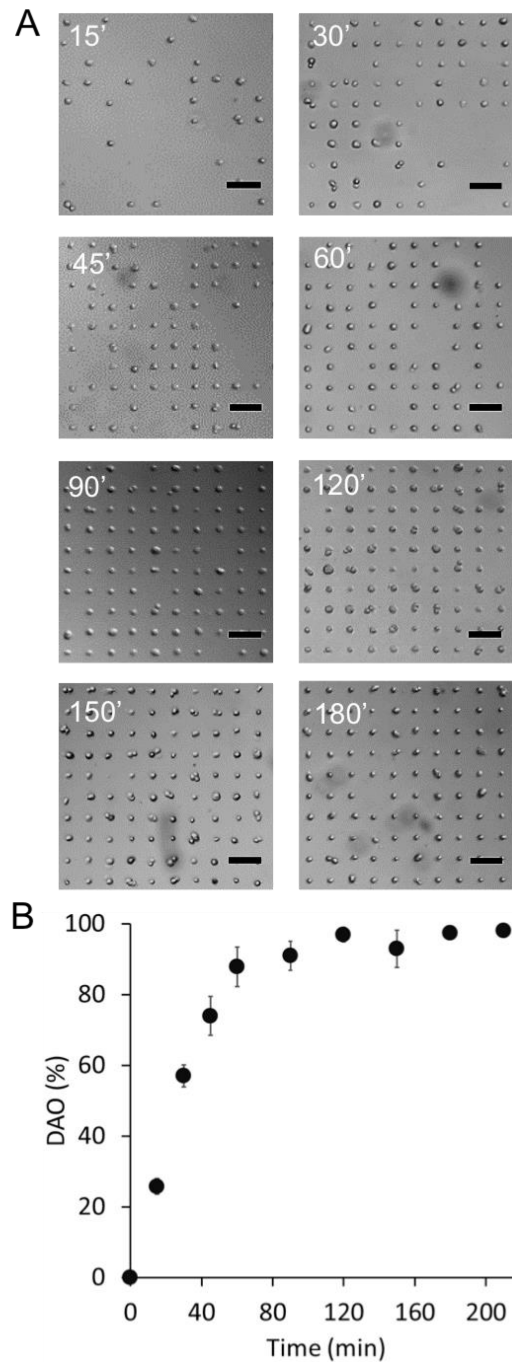
223 **3. Results**

224 3.1. *Monitoring cell adhesion and detachment to protein substrates with single cell*
225 *resolution.*

226 Fibronectin (FN) is a protein of high molecular weight from the Extracellular Matrix (ECM),
227 which plays a crucial role in cell adhesion, as it binds to integrins located in the cell
228 membrane. On the other hand, primary human hair follicle Mesenchymal Stem Cells (hHf-
229 MSCs), are adherent cells that express a number integrin molecules like α1, α2, α3, α4, α5,
230 αv, β1, β3 and β4, some of which have been previously reported to be involved in MSC
231 adhesion (17). In order to demonstrate the suitability of micro-patterned SCADA substrates
232 to monitor cellular death, first the adhesion and detachment kinetics of hHf-MSCs to a FN-

233 SCADA substrate were monitored. Arrays of 20 μm FN dots with inter dot space of 50 μm
234 were created on polystyrene (PS) cellular culture dishes by micro-contact printing, and the
235 remaining surface of the dish was blocked with Bovine Serum Albumin (BSA) to avoid non-
236 specific cellular adhesion. To evaluate the homogeneity of the composition of the protein
237 dot array, the fluorescence intensity and the diameter of 3.300 dots were analysed within a
238 single substrate. The coefficient of variation (CV) for fluorescence intensity and dot diameter
239 values were of 6 % and 14 % respectively, confirming the homogeneity of the dot
240 composition and their shape along the substrate. 1 mL of 100.000 cell/mL suspension was
241 added to each patterned well, and kept incubating at 5 cycles per minute (steep angle
242 rocking). To determine adhesion kinetics, the occupancy of the dot array (DAO) was
243 measured after 15', 30', 45', 1 h, 1.5 h, 2 h, 2.5 h, 3 h and 3.5 h, taking optical microscopy
244 images of the substrates at each time point, and calculating the ratio of occupied binding
245 dots. As shown in **Figure 2**, cells started to attach to FN dots in a few minutes, and just after
246 30 minutes of incubation, there was more than 50% occupancy of the protein dot array. After
247 2 h of incubation, hHF-MSCs reached the adhesion plateau with a DAO of 97%. Controlled
248 cellular detachment was induced by exposing the cell-saturated substrates to a solution of
249 PBS lacking Ca^{2+} and Mg^{2+}. Integrins have 3 to 5 binding sites for divalent cations in each
250 heterodimer. Different cations play different roles; they may act as adhesion promoters
251 inducing ligand binding, but also as antagonists inhibiting adhesion (18). It is known that the
252 absence of Ca^{2+} and Mg^{2+} in the media affects the equilibrium between the active and the
253 inactive forms of integrins, and promotes cellular detachment without damaging the cells
254 (19). hHF-MSCs detached from FN dots slowly in the first 40 minutes, whereas from that
255 time on, their detachment kinetics increased significantly. After 2 hours in PBS, cellular
256 release from the surface became slower, until every adhered cell was detached, after 4
257 hours (**Figure 3**). Flow cytometry confirmed that more than 60 % of detached cells remained

258 alive, confirming the controlled detachment triggered by the lack of Ca^{2+} and Mg^{2+}. This
259 experiment proved the capability of this technique to perform a label-free, quantitative and
260 dynamic monitoring of cellular detachment with single cell resolution by using optical
261 components.

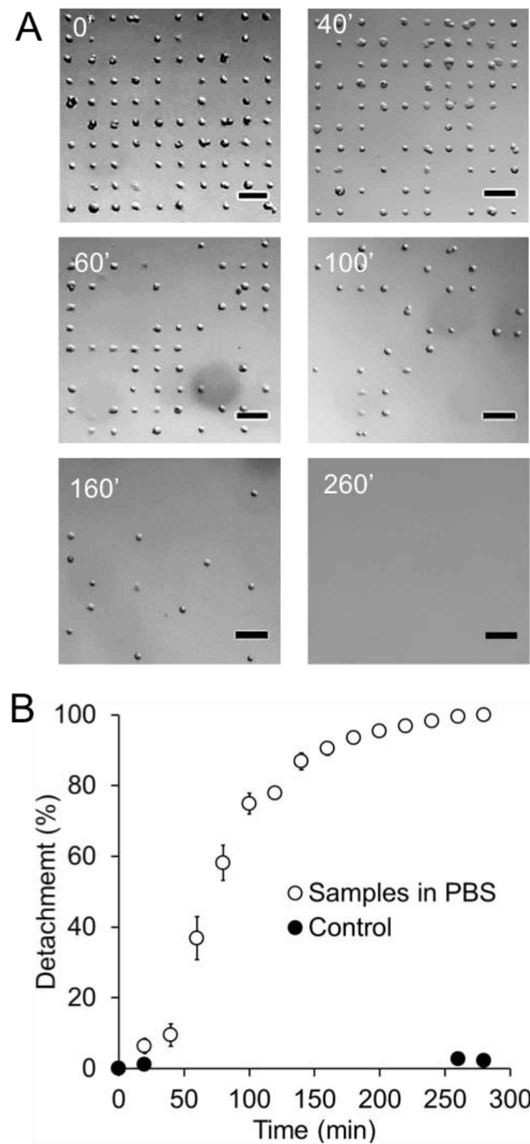


262

263 ***Figure 2.*** *A)* Optical microscopy, brightfield, images *of single cell adhesion to the* dot array
264 *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*

265 *and (viii) 3.5 h. B) Dot Array Occupancy (DAO)* versus time. Scale bar corresponds to 100

266 µm. Error bars correspond to the standard deviations (n=3)



267

268 **Figure 3.** A) Optical microscopy images of single cell arrays at different times in presence

269 of PBS: (i) at time 0, (ii) 40 min, (iii) 60 min, (iv) 100 min, (v) 160 min and (vi) 260 min. Scale

270 bar corresponds to 100 µm. B) Detachment of hHF-MSCs versus time of exposure to PBS.

271 Error bars correspond to the standard deviations (n=3)

272

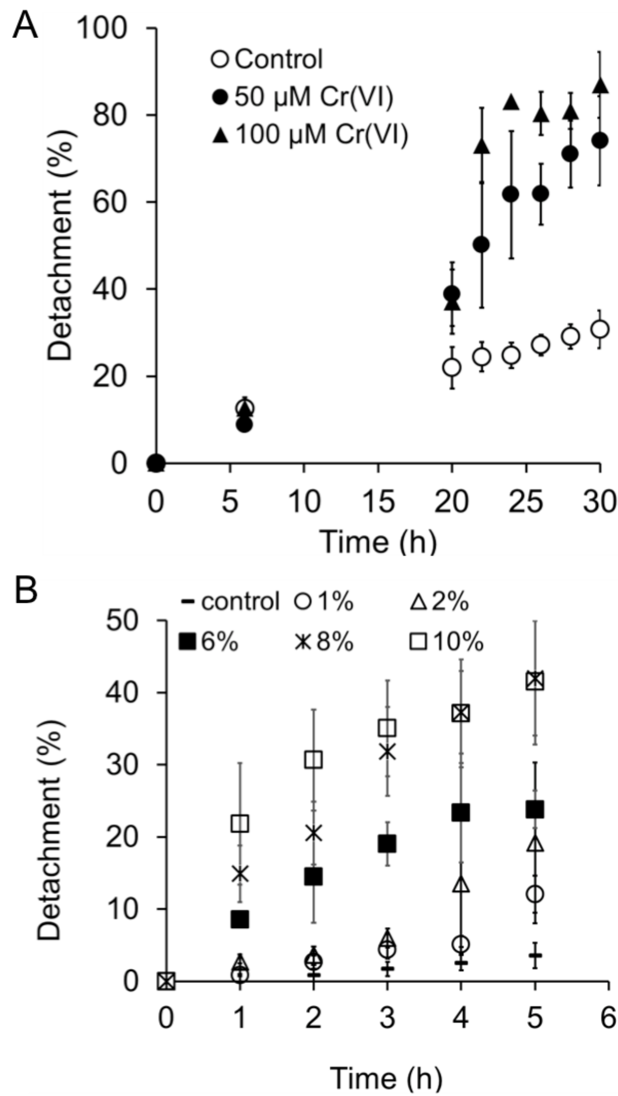
273

274

275 ### *3.2. Monitoring cytoxicity of K_2CrO_4 and DMSO measured by label free SCADA viability*
276 *test*

277 Potassium chromate is a strong oxidant agent, which is considered to be highly toxic, while
278 DMSO is an organosulfur compound widely used for biological applications. DMSO
279 produces apoptosis *in vitro* when it is used in higher concentrations than 10% (v/v), for this
280 reason it is usually used at lower concentrations for cell culture (20).
281 Cell saturated SCADA substrates were incubated with solutions of 50 μM and 100 μM of
282 K_2CrO_4 for 30 hours. The data obtained showed increasing cellular detachment with the
283 exposure time and the concentration of the toxic after six hours of incubation **(Figure 4)**.
284 After 20 hours in presence of 50 and 100 μM of K_2CrO_4, there was a 20 % of cellular
285 detachment for control samples, which increased up to 74 % and 87 % for the case of 50
286 and 100 μM of K_2CrO_4 respectively. Instead, control samples showed only a maximum
287 detachment value of 30% after 30 hours. Cr(VI) has been previously reported to cause
288 replication stress (21), which can lead to apoptosis or programmed cellular death (22,23). This
289 is in agreement with our results showed in this paper, as significant detachment could only
290 be observed after 6 hours of incubation with the toxic.
291 To evaluate the cytotoxicity of DMSO, the effect of, 1, 2, 6, 8 and 10% (v/v) DMSO was
292 measured on cells adhered to FN-SCADA substrates for 24 h. Our measurements showed
293 an increasing detachment of cells with time of exposure to DMSO. When cells were treated
294 with 8% and 10% of DMSO (v/v), after 24 hours 100% of the cells were detached from the
295 substrate. In general, faster detachment kinetics were observed for higher concentrations of
296 DMSO. In both cases, when the cytotoxicity of K_2CrO_4 and DMSO were evaluated, trypan
297 blue viability test showed that all the cells that remained adhered to the substrate were alive,
298 revealing a good correlation between cellular death and detachment. To the best of our

299 knowledge, the effect of DMSO concentrations below 10% for hHfF MSCs has not been
300 precisely quantified before in terms of cellular death.
301



302

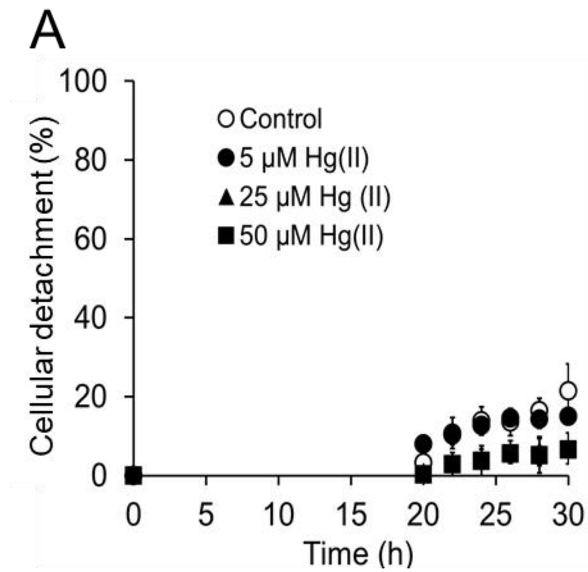
303 **Figure 4.** Cellular detachment kinetics in presence of A) 50 and 100 μM K_2CrO_4 for 30 hours
304 and B) 1, 2, 4, 6, 8 and 10 % of DMSO for 25 hours. Error bars correspond to the standard
305 deviations (n=3)
306

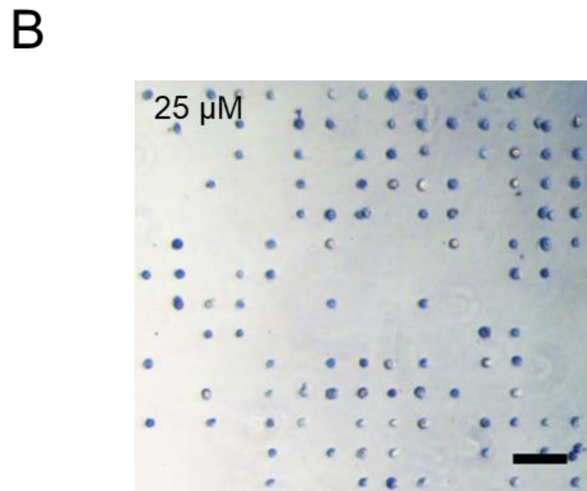
307 *3.3. Cytotoxicity colorimetric SCADA viability test*

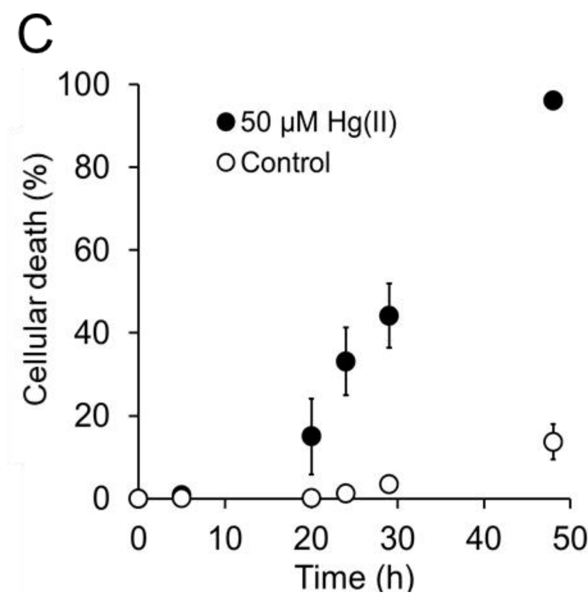
308 $HgSO_4$ is a toxic compound that decomposes producing mercury (Hg^{2+}) and sulfur oxides
309 (24). This divalent cation promotes stable adhesion of hHF-MSCs on FN. In this work, this

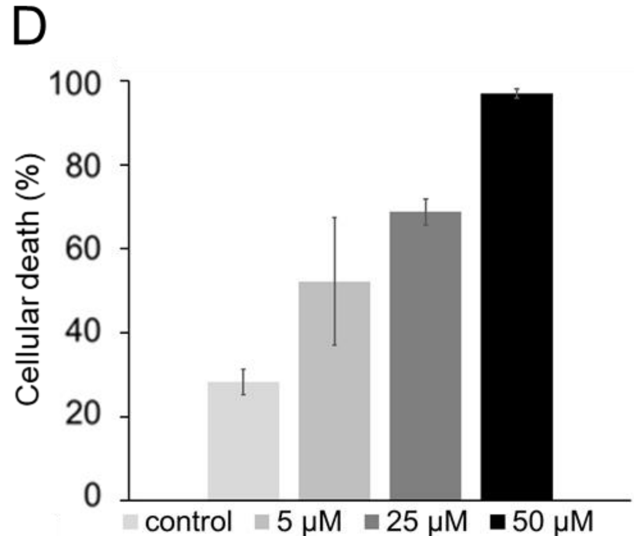
310 model was used to monitor cell death by a colorimetric assay using the binary counting
311 board of occupied/vacant. Cell saturated SCADA substrates were exposed to $HgSO_4$
312 solutions of 5, 25 and 50 μM for 30 hours. It was observed that the presence of $HgSO_4$ did
313 not alter much cell attachment compared to control samples (**Figure 5**). To monitor cell
314 death in this case, trypan blue was used, a life/death marker that penetrates the cellular
315 membrane of dead cells, staining them in blue. As it cannot enter through healthy cellular
316 membranes, living cells remain uncoloured. Cell saturated FN-SCADA substrates were
317 exposed to 50 μM of $HgSO_4$ for 48 hours, and trypan blue was added at different time points
318 to measure cell viability. The quantification of blue and non-coloured cells on the substrate
319 at each time point showed an increasing number of dead cells with the time of exposure to
320 the toxic compound **(Figure 5)**. In a different experiment, cells adhered to FN-SCADA
321 substrates were exposed to solutions of 5 μM, 25 μM and 50 μM of $HgSO_4$ for 48 hours, and
322 the trypan blue assay also showed an increasing rate of cell death with the concentration of
323 $HgSO_4$, confirming its cytotoxicity.
324

325









326 **Figure 5.** A) Cellular adhesion kinetics of hHF MSCs in presence of 5, 25 and 50 μM of
327 $HgSO_4$. B) Optical microscopy image of FN-SCADA substrates with hHF MSCs 48 h after
328 incubation with 25 μM of $HgSO_4$ at 10x. Scale bar corresponds to 100 μm. C) Graphical
329 representation of cellular death during 48 hours in presence of 50 μM of $HgSO_4$. D) Plot of
330 cell death after 48 hours in presence of 5, 25 and 50 μM of $HgSO_4$. Error bars correspond to
331 the standard deviations (n=3)

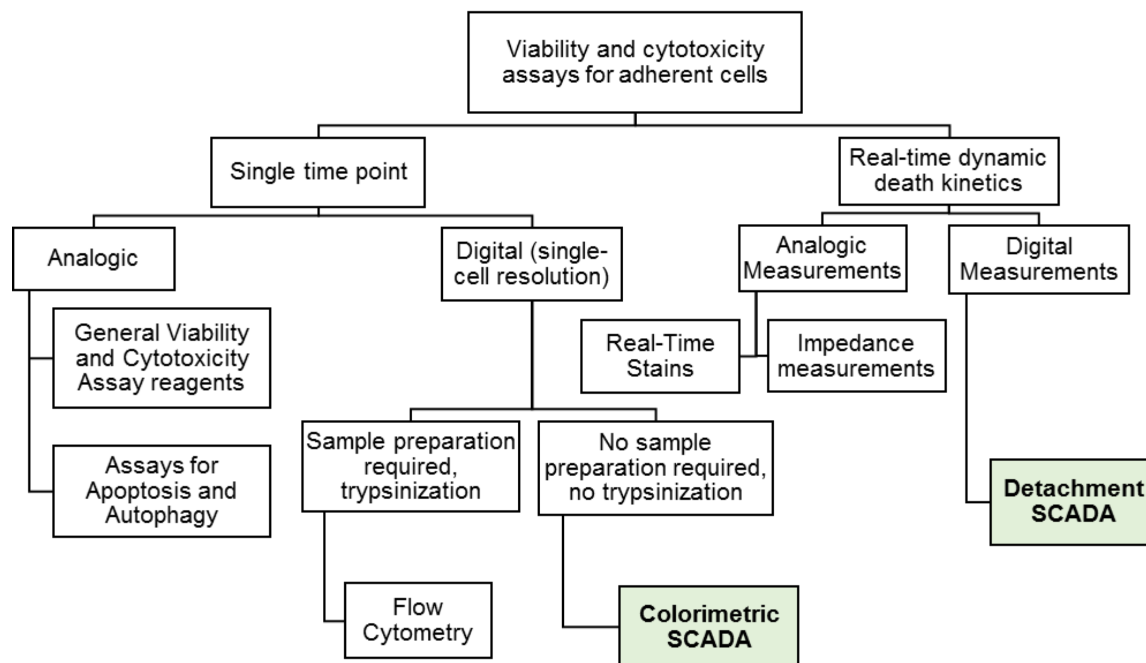
332

333

4. Discussion and conclusions

SCADA substrates enabled the measurement of the adhesion kinetics of hHF MSCs to fibronectin as well as the kinetics of controlled cellular detachment from fibronectin induced by the lack of Ca^{2+} and Mg^{2+} in cell culture media with single cell resolution. The toxic effect of DMSO and K_2CrO_4 on hHF MSCs was successfully monitored measuring cell detachment rate in a label free mode. In a different case, the toxic effect of $HgSO_4$ was measured, and despite of the fact that it inhibited cellular detachment from the surface, its cytotoxicity was monitored using Trypan Blue life/death assay directly on the SCADA substrates. The precision of the SCADA system is based on a binary counting mode for the quantification of cellular adhesion, detachment and staining, which is enabled by an array of adhesive dots for individual cells. 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 0/1 code. 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.

There are a number of cytotoxicity test 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.



358

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

363 In contrast with current available cytotoxicity tests based on analogical signals like light or
364 impedance intensity, SCADA is based on digital counting, providing the highest resolution,
365 single cell resolution. Besides, compared to flow cytometry, SCADA methodology allows
366 dynamic and real time measurements, because it avoids the time gap between the target
367 time when the toxic is added and the time in which the cellular death measurements are
368 performed. SCADA assays require only a patterned substrate area of less than 1 cm^2, and
369 a reduced number of cells, ranging from hundreds to thousands of cells, while other
370 techniques normally use more than 10.000 cells per measurement. Nonetheless, the
371 number of cells analysed in SCADA could be increased to a million cells if needed, by using
372 larger patterned areas of the substrate. For this proof of concept, the patterned substrates
373 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. The readout of this assay requires only cheap 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.

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