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## Soft, skin-interfaced microfluidic systems with integrated immunoassays, fluorometric sensors and impedance measurement capabilities

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### ► To cite this version:

Sungbong Kim, Boram Lee, Jonathan T Reeder, Seon Hee Seo, Sung-Uk Lee, et al.. Soft, skin-interfaced microfluidic systems with integrated immunoassays, fluorometric sensors and impedance measurement capabilities. *Proceedings of the National Academy of Sciences of the United States of America*, 2020, 117 (45), pp.27906-27915. 10.1073/pnas.2012700117 . hal-03414153

**HAL Id: hal-03414153**

**<https://hal.science/hal-03414153v1>**

Submitted on 4 Nov 2021

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1 **Soft, skin-interfaced microfluidic systems with integrated immunoassays,**  
2 **fluorometric sensors and impedance measurement capabilities**

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43

44 **Classification**

45 Engineering

46

47 **Keywords**

48 Healthcare, Soft materials, Epidermal devices, Sweat cortisol, Galvanic skin response

49

50 **Author Contributions**

51 S.K., B.L., J.T.R., S.H.S., R.G., J.K., P.V.B. and J.A.R. led development of concepts, organization  
52 of system, designed experiments and interpreted results. B.L. (MD PhD) designed human tests,  
53 and provided medical consultations, advice and discussion. S.K., B.L., S.H.S., J.K. led over all  
54 experiments, fabrications, integrating of subsystems, and finishing with support from J.T.R., A.H.-  
55 F., H.J., G.L., Y.S.O., A.J.A, S.P.L., J.B.M., M.-H.S., S.S.K., S.J., G.P. and S.H. Also, S.-U.L.,  
56 Y.S.O., I.P. performed 3D mechanical modeling and analysis, Y.S.O. provided original idea of  
57 skeletal microfluidics and J.S., Y.S., H,-I,J. provided immunoassay and fluorescence assay  
58 information and discussion. S.K., B.L., J.T.R., S.H.S., S.-U.L., A.H.-F., Y.S., Y.O., A.J.A, S.P.L.,  
59 J.B.M., S.J., R.G., J.K. P.V.B and J.A.R led paper writing.

60

61 **Competing interests**

62 J.A.R., J.T.R., Y.S. and S.K. are inventors on a Patent Cooperation Treaty (PCT) patent by the  
63 board of trustees of Northwestern University (PCT/US2018/035661), and J.A.R., J.T.R., S.K., Y.S.  
64 and A.H-F. are inventors on a PCT patent by the board of trustees of Northwestern University  
65 (PCT/US2018/035738). R.G., A.J.A, J.B.M., S.P.L., J.A.R. are cofounders of Epicore Biosystems  
66 Inc., which pursues commercialization of epifluidic devices. All other authors declare that they have  
67 no competing interests.

68

69 **This PDF file includes:**

70 Main Text  
71 Figures 1 to 5

72

73 **Abstract**

74           Soft microfluidic systems that capture, store and perform biomarker analysis of microliter  
75 volumes of sweat, *in situ*, as it emerges from the surface of the skin, represent an emerging class  
76 of wearable technology with powerful capabilities that complement those of traditional biophysical  
77 sensing devices. Recent work establishes applications in real-time characterization of sweat  
78 dynamics and sweat chemistry in the context of sports performance and healthcare diagnostics.  
79 This paper presents a collection of advances in biochemical sensors and microfluidic designs that  
80 support multimodal operation in monitoring of physiological signatures directly correlated to  
81 physical and mental stresses. These wireless, battery-free, skin-interfaced devices combine,  
82 lateral flow immunoassays for cortisol, fluorometric assays for glucose and ascorbic acid (vitamin  
83 C), and digital tracking of skin galvanic responses. Systematic bench-top evaluations and field  
84 studies on human subjects highlight the key features of this platform for continuous, non-invasive  
85 monitoring of biochemical and biophysical correlates of stress state.

86 **Significance Statement**

87           Skin-interfaced, wireless devices for clinical-grade monitoring of physiological parameters  
88 are of growing interest for uses that range from delivering high quality care. This paper introduces  
89 a multifunctional skin-mounted microfluidic platform for capture and biomarker analysis of microliter  
90 of sweat that is a biofluid has potential relevance in biophysical sensing and healthcare. The focus  
91 is on colorimetric and digital assessments of a collection of parameters related to stress, including  
92 concentrations of vitamin C, cortisol and glucose in sweat, along with quantitative measurements  
93 of sweat rate and galvanic skin response. The results represent important additions to a portfolio  
94 of emerging capabilities in skin-interfaced technologies for physiological monitoring, with particular  
95 relevance to conditions that follow from unhealthy levels of physical and mental stress.

96

97 **Main Text**

98  
99           Soft, wearable microfluidic systems with capabilities in colorimetric, fluorometric and  
100 electrochemical sensing of sweat biomarkers offer a range of modalities for tracking performance,  
101 nutrition, wellness and health (1-5). These technologies exploit the rich mixture of solutes,  
102 metabolites, hormones of eccrine sweat and its non-invasive extraction directly from pores on the  
103 surface of the skin (6-10). A key requirement for the broad adoption of devices for sweat sensing  
104 is in contamination-free capture of precise volumes of sweat and *in situ* quantitative analysis of  
105 multiple biomarkers with relevance to muscle fatigue, dehydration, cystic fibrosis and others. An  
106 important and relatively unexplored frontier focuses on capabilities that support qualitatively  
107 expanded domains of application, such as those in tracking biochemical correlates of physical and  
108 mental stresses, and other aspects of cognitive status. This area represents the main focus of the  
109 results reported here.

110           Conventional techniques for sweat analysis rely on absorbent pads that adhere to the skin  
111 and require subsequent removal, special handling, bench-top centrifugation, and extraction of  
112 sweat for off-site analysis (11-13). These lab-based strategies are incompatible with real-time  
113 monitoring in field settings due to the need for expensive and bulky analysis equipment. Recent  
114 developments in advanced, soft forms of microfluidic technologies with integrated chemical and  
115 electrochemical sensors serve as the foundations for opportunities in real-time monitoring of  
116 various sweat biomarkers and tracking of sweat loss and local rate (3,14-16). Such devices are  
117 thin and flexible, thereby allowing conformal, water-tight coupling to the skin in clinical, athletic, and  
118 real-world environments. Related designs that incorporate capillary burst valves and mechanically  
119 reinforced stiffening materials (e.g. skeletal designs) enable time sequential analysis (i.e.  
120 chronosampling) of multiple sweat biomarkers, and application in demanding scenarios that involve  
121 physical impacts (17-19), respectively. In other demonstrations, surface coated electrodes with  
122 antibody- and enzyme-based assays capture information about cortisol and related biochemicals  
123 in a continuous mode of operation, although without field studies to demonstrate robustness of  
124 operation required for practical applications (20-23).

125           Simultaneous, reliable analysis of species such as cortisol, together with vitamin C and  
126 glucose, has the unique potential to yield insights into transient states of physical and mental stress.  
127 Cortisol release from the adrenal glands occurs in response to cognitive and physical stressors.  
128 This release activates the sympathetic nervous system (24) and triggers a complex chain of  
129 biochemical responses that lead to an increase in energy production (25). In particular, cortisol  
130 secretion in response to stress leads to elevated levels of glucose for muscle groups to consume  
131 in “fight or flight” scenarios. Increased cortisol levels over prolonged time periods, however, have  
132 been linked to conditions such as obesity, depression, hypertension, and diabetes (26).

133 Supplementary intake of vitamin C can counteract these harmful effects by boosting the immune  
134 response and attenuating cortisol levels (27). A desire to understand the complex relationship  
135 between cortisol, glucose, and vitamin C that define dynamic stress responses, motivates the  
136 development of devices for non-invasive monitoring of these stress-related biomarkers, as a means  
137 for establishing counteractive interventions.

138 This paper reports technologies that allow measurements of multiple stress-related  
139 biomarkers in battery-free, wireless skin-interfaced device platforms. The designs described here  
140 include skeletal microfluidic networks with integrated quantitative immunoassays for cortisol and  
141 fluorescence assays for glucose and vitamin C, along with features that allow for continuous  
142 sensing of sweat rate and conductivity using galvanic skin response (GSR). Demonstrations in  
143 bench-top studies and in field trials on human subjects highlight unique capabilities in multimodal  
144 and non-invasive monitoring of stress during exercise and at rest in real-world settings.

145  
146

## 147 **Results and Discussion**

148  
149

150 **Soft, skin-interfaced skeletal microfluidic systems with lateral flow immunoassays and**  
151 **digital wireless measurement capabilities.** Skin interfaced systems with integrated  
152 immunoassays for sweat cortisol, fluorescent assays for glucose and ascorbic acid (vitamin C), and  
153 with electrochemical sensors, support an important range of capabilities for analysis of sweat  
154 biomarkers and sweat dynamics related to stress. These multimodal features in sensing exploit  
155 ruggedized microfluidic structures formed using a high modulus (~1 GPa), ultra-violet (UV) curable  
156 polyurethane (NOA81) embedded in a low modulus (~30 kPa; Ecoflex™, Smooth-On Inc., PA)  
157 silicone polymer matrix (28, 29). Fig. 1A shows an exploded view of this ‘skeletal’ microfluidic  
158 design. Compared to systems based on traditional elastomers, these polyurethane-based  
159 microfluidic structures greatly reduce the rate of evaporation of collected and stored sweat (29, 30)  
160 (*SI Appendix*, Fig. S1A and B) and serve as fluidic connections for embedded lateral flow  
161 immunoassays (LFIA). *SI Appendix*, Fig. S1C and D shows a schematic illustration and an optical  
162 image of the skeletal microfluidic channels, respectively. The microfluidic channel stores ~ 70  $\mu$ L,  
163 and electrodes within these channels establish contact with sweat for continuous, resistive  
164 measurements of sweat rate. Additional structures define micro-reservoirs and capillary burst  
165 valves for fluorescence-based measurements of glucose and ascorbic acid. A medical-grade skin  
166 adhesive layer with patterned openings attaches the device to the skin and defines access points  
167 for collection of sweat directly from skin pores, at positions aligned to inlets on the backside of the  
168 device (16). *SI Appendix*, Fig. S2 shows bench-top results of a device filling with artificial sweat at  
169 a rate of ~2  $\mu$ L/min. An integrated system with electrodes, serpentine microchannels (600  $\mu$ m  
width, 400  $\mu$ m depth), and an LFIA for cortisol measurement appears in Fig. 1B and C. Fig. 1D

170 illustrates the design of the microfluidic assembly for fluorescence-based glucose and ascorbic acid  
171 assays.

172 Measurements of sweat loss and GSR exploit a collection of ultrathin electrodes (3- $\mu$ m  
173 thick) defined photolithographically using a tacky formulation of polydimethylsiloxane (PDMS; 30:1  
174 mixture of base to curing agent; Sylgard 184, Dow Corning; Midland, MI) as a temporary carrier.  
175 These patterned electrodes bond to the polyurethane in a UV curing process as shown in *SI*  
176 *Appendix*, Fig. S3A. *SI Appendix*, Fig. S3B presents images of electrodes after UV curing (top)  
177 and of a representative device with integrated electrodes (bottom). *SI Appendix*, Fig. S4 shows  
178 results from a simple peel test (Mark-10 ESM1500; ABQ Industrial L.P., TX; *SI Appendix*, Fig. S4A-  
179 C) that indicate levels of adhesion between the electrodes and the NOA81 substrate are  $\sim$ 6 times  
180 stronger than those associated with bonding to a tacky piece of PDMS (*SI Appendix*, Fig. S4D and  
181 E). Aligned bonding of the relief side of the prepared structure to the electrode layer yields an  
182 enclosed skeletal microfluidic channel system with integrated electrode interfaces (*SI Appendix*,  
183 Fig. S3C). The electrodes integrate along the serpentine microchannels with direct electrical  
184 interfaces to the sweat (Fig. 1B) as illustrated in *SI Appendix*, Fig. S5A. The system includes  
185 reference electrodes (① in *SI Appendix*, Fig. S5A), tracking electrodes (④ in *SI Appendix*, Fig.  
186 S5A), and a counter electrode (② or ③ in *SI Appendix*, Fig. S5A). *SI Appendix*, Fig. S5B shows  
187 magnified optical images of the reference (top) and tracking electrodes (bottom).

188 The packaging scheme highlighted in *SI Appendix*, Fig. S3D and *SI Appendix*, Fig. S6A  
189 involves steps to embed the microfluidic structure within silicone (Ecoflex<sup>TM</sup>; 1:1 mixing, cured at  
190 room temperature for 12 h). A laser cutting process defines the perimeter of the resulting system  
191 (Fig. 1E, *SI Appendix*, Fig. S3D, *SI Appendix*, Fig. S7B and *SI Note* 1). Fig. 1E shows the device  
192 during mechanical twisting and bending. Fig. 1F and *SI Appendix*, Fig. S6C-E present results of  
193 finite element analysis (FEA) of the associated mechanics (*SI Note* 2). The serpentine geometries  
194 of the microfluidic channels and the low modulus, stretchable silicone matrix ( $\sim$ 30 kPa) facilitate  
195 high levels of elastic deformations (31-33) (*SI Appendix*, Fig. S6C-E).

196

197 **Lateral flow immunoassay for cortisol.** The cortisol immunoassay relies on a competitive  
198 reaction that exploits anti-mouse-IgG (Anti-IgG) antibody, cortisol conjugated bovine serum  
199 albumin (cortisol-BSA) and gold nanoparticles (AuNPs; *SI Appendix*, Fig. S7A) with conjugated  
200 anti-cortisol antibodies (ACA) (34-36). *SI Appendix*, Fig. S7B and C schematically illustrates the  
201 reagents and immunoreactions. The ionic affinity of the hydrophobic surfaces of the AuNPs  
202 facilitates preparation of ACA-AuNP conjugates via spontaneous reaction of ACA and AuNP at  $\sim$   
203 pH 7.2,  $\sim$ 23°C and 35% humidity. The immunoassay initiates as approximately 90% of collected

204 sweat (~60  $\mu$ L) from the main channel reaches the conjugation pad, and cortisol in the sweat  
205 conjugates with the ACA-AuNP (cortisol-ACA-AuNP). Sweat then wicks along the nitrocellulose  
206 (NC) membrane to launch immunoreactions that occur along control and test lines defined on the  
207 membrane. The control line screens uncoupled ACA-AuNP by immobilizing the ACA active sites  
208 via cortisol-BSA. The test line senses cortisol-ACA-AuNP quantitatively as a result of binding to  
209 anti-IgG (34) (*SI Appendix*, Fig. S7B and C). Since there are multiple binding sites per AuNP, the  
210 sensitivity and dynamic range of the assay depend on the number of AuNPs, the number of binding  
211 sites per AuNP, the total amount of cortisol-BSA, and the concentration of sweat cortisol.

212 Fig. 2A highlights the zeta-potential as a function of ACA concentration (0.02, 0.2, 2 and  
213 20 mg/mL ACA). The error bars are mean values across 3 samples with standard deviations for  
214 each concentration condition for conjugation of AuNPs (diameters of 30 nm, with highest optical  
215 density at ~550 nm wavelength) as shown in *SI Appendix* Fig. S7A. Increasing the concentration  
216 of ACA increases the surface charge, until saturation at ~2 mg/mL ACA. These measurements  
217 indicate that ~0.5 mg/mL ACA (~ -20 mV of zeta-potential) is a good choice for conjugation of ACA  
218 and AuNPs (37, 38). Fig. 2B shows the absorbance spectrum for ACA-AuNP samples for various  
219 times of conjugation. The results reveal the time dependence of AuNP aggregation, and the  
220 corresponding time required for saturation of the color response (39, 40) (*SI Appendix*, Fig. S8A).  
221 Insufficient conjugation time produces samples with absorbance lower than those processed in an  
222 optimized manner (3 min, as in Fig. 2B). Fig. 2C shows that the peak absorbance occurs at ~280  
223 nm, a wavelength range where aromatic groups in the amino acid residues and antibodies absorb  
224 strongly (40-42). Transmission electron microscope (TEM) images (*SI Appendix*, Fig. S8B)  
225 compare AuNPs before (left) and after conjugation (right), and the image after conjugation (right)  
226 shows development of ACA on the AuNP surface. *SI Appendix*, Fig. S8C shows the effects of  
227 physiologically relevant changes in sweat pH (pH 5.0, 6.0, 7.0 and 8.0) on zeta-potentials for  
228 samples of ~0.5 mg/mL ACA conjugated with 30-nm AuNP. The shift in zeta-potentials is ~ -25  
229 mV to ~ -41 mV for changes in pH from 5.0 to 8.0 that is based on that the carboxyl groups and  
230 hydroxyl groups are ionized in the shifting alkaline condition (43).

231 The lateral flow strip consists of a conjugation pad (Glass fiber; GFB-R7L; mdi Membrane  
232 Technologies, Inc., PA; *SI Appendix*, Fig. S9A) and a NC membrane (10- $\mu$ m pore size) with color  
233 development at the cortisol-BSA control line (*SI Appendix*, Fig. S9B). The absorbent pad confines  
234 the reaction system within the NC membrane to ensure rapid and accurate immunoassay reactions.  
235 Selection of the membrane material and pore size follow considerations based on the Lucas-  
236 Washburn model, according to (44, 45);

237 
$$L^2 = \gamma r t \cos\theta / 2\eta \quad (\text{Eq. 1}),$$



238 where  $L$  is the absorption distance,  $\gamma$  is the surface tension,  $r$  is the pore radius,  $\theta$  is the contact  
239 angle between the membrane material and the solution,  $t$  is the time and  $\eta$  is the dynamic viscosity  
240 of the solution. Once the collected sweat activates LFIA reaction from conjugation pad, the overall  
241 reaction time remains constant, consistent with the Lucas-Washburn model and independent of  
242 sweat rate. *SI Appendix*, Fig. S9C-E show the process for assembly of the LFIA strip and optical  
243 images before and after laser cutting. A dispensing process delivers cortisol-BSA and anti-IgG to  
244 the control and test lines, respectively. The immunoassay design and detection range depend on  
245 the amount of immobilized cortisol-BSA on the control line and the active surface areas of both the  
246 control and test lines. Color development tests on the control line as a function of concentration of  
247 cortisol-BSA determine the optimal quantity of cortisol-BSA (Fig. 2D). Fig. 2D shows the onset of  
248 color saturation at  $\sim 7$  mg/mL cortisol-BSA with the assembled test strip (rectangular; 5 cm x 0.5  
249 cm) that 1 mL of prepared 0.8 OD ACA-AuNP is added to the unprocessed conjugation pad (*SI*  
250 *Appendix*, Fig. S9D; 6 cm x 1 cm of glass fiber) in the assembly process. Accurate colorimetric  
251 evaluation of the LFIA involves analysis of images captured with a smartphone, after correcting for  
252 ambient lighting conditions (e.g. direct sun light, shade, indoor lighting, transmission properties  
253 through the polyurethane micro-channel; Eq. S1) and with the NC membrane surface as a white  
254 reference (*SI Note 3*) (5, 46).

255 Fig. 2E shows an image of completed LFIA strip and *SI Appendix*, Fig. S10A shows the  
256 strip integrated in a device for measurements across the physiological range of cortisol  
257 concentrations at the test line (Fig. 2F; 5, 10, 30, 60 and 100 ng/mL; 50  $\mu$ g/mL Anti-IgG immobilized  
258 at the test line). Pictures of the LFIA strips after 10 min of color development appear in *SI Appendix*,  
259 Fig. S10B. Tests with volunteer subjects indicate the ability to measure cortisol concentrations  
260 accurately using this approach, as an alternative to enzyme-linked immunosorbent assay (ELISA)  
261 tests that is reliable bench-top cortisol assay, as shown in Fig. 2G (47).

262

263 **Fluorescence-based assays for glucose and ascorbic acid.** The device also supports  
264 fluorescence-based assays for glucose and ascorbic acid. A pair of reservoirs connected by  
265 microchannels and capillary burst valves enable time-sequential sampling of sweat for these  
266 measurements. The passive valve geometries have lateral dimensions ( $\sim 50$   $\mu$ m) that are  
267 significantly smaller than those of the microchannels leading into the reservoirs (150  $\mu$ m). The  
268 burst pressure mechanism follows from the Laplace-Young equation (Eq. 2) according to:

269 
$$\text{Burst Pressure} = -2\sigma \left[ \frac{\cos\theta_I^*}{b} + \frac{\cos\theta_A}{h} \right] \quad (\text{Eq. 2})$$

270 where  $\sigma$  is the surface tension of liquid,  $\theta_A$  is the contact angle of the channel,  $\theta_I^*$  is the min [ $\theta_A + \beta$ ;  
271  $180^\circ$ ],  $\beta$  is the diverging angle of the channel,  $b$  and  $h$  are the width and the height of the diverging

272 section, respectively (30, 48, 49). *SI Appendix*, Fig. S11A illustrates the overall design of this  
273 network of channels, assays, and the capillary burst valves where 'Valve#1' and 'Valve#2' have  
274 diverging angles of 90° and 120°, respectively. Magnified optical images for the valves are shown  
275 in *SI Appendix*, Fig. S11B. Tailoring the burst pressures for these valves ensures ordered routing  
276 of sweat as it fills into the reservoirs. A separate set of circular reservoirs not connected to the  
277 microfluidic network serve as fluorescence reference markers prefilled with fluorescent dye (5  
278 mg/mL Tetramethylrhodamine, Ethyl Ester, Pechlorate; TMRE; Thermo Fisher Scientific, Waltham,  
279 MA) (50).

280 *SI Appendix*, Fig. S12A and B summarize reactions that involve glucose and ascorbic acid  
281 with glucose oxidase (GOx) and ascorbic acid oxidase (AOx) enzymes, respectively. The oxidation  
282 reactions for both substrates generate hydrogen peroxide, and excess activity of horseradish  
283 peroxidase (HRP) leads to reduction of a fluorometric probe (OxiRed™) to form resorufin as the  
284 basis for a fluorescence signal (Fig. 3A;  $\lambda_{excitation}$ : ~550 nm wavelength and  $\lambda_{emission}$ : ~600 nm  
285 wavelength) with magnitude that depends on the concentration (51) (*SI Appendix*, Fig. S12C). *SI*  
286 Note 4 summarizes the details of enzymes preparation for glucose and ascorbic acid assays. *SI*  
287 *Appendix*, Fig. S12D and E shows the effect of pH on the activity of GOx and AOx, respectively.  
288 An apparatus with integrated excitation and emission filters attaches to a smartphone to facilitate  
289 rapid measurement of the fluorescence *in situ* (49) (Fig. 3B). The emission filter passes only light  
290 with wavelengths longer than 610 nm. *SI Appendix*, Fig. S12F shows the key features of this  
291 module and its coupling to a smartphone imager. This setup facilitates capture of fluorescence  
292 signals, as measures of the concentration of target substrates (*i.e.* glucose and ascorbic acid). Fig.  
293 3C features signals from glucose and ascorbic acid relative to those from the TMRE reference  
294 reservoir. Calibration involves first analyzing the signal intensity from each reservoir and then  
295 normalizing these results by the intensity of TMRE (49, 52) (Eq. S2). The depth of each  
296 microreservoir controls the dynamic range of the fluorescence signal, according to the Beer-  
297 Lambert law (53). Fig. 3D shows the effect of the silicone packaging on the fluorescent signal at  
298 0:10, 1:9, 3:7 and 10:0 ratios (0%, 10%, 30% and 100%, respectively) of black and white pigments  
299 mixed in uncured Ecoflex at ~5% (w/w). Fig. 3E and F exhibit examples that the fluorescence  
300 intensity increases for glucose and ascorbic acid assays in a linear manner with relevant range of  
301 concentrations.

302

303 **Battery-free, wireless electronic interface for readout of sweat rate and galvanic skin**  
304 **response.** Fig. 4A highlights the wireless electronic module, which consists of three electrodes  
305 (sweat rate, skin conductance, and sweat reference), a near field communication (NFC)  
306 microcontroller (RF430, ISO/IEC 15693, ISO/IEC 18000-3; Texas Instruments, Dallas, TX) and an

307 RF antenna. The microcontroller receives power wirelessly (*SI Appendix*, Fig. S13A-D) from an  
 308 NFC-enabled device such as smartphone (16, 54, 55). The electrodes deliver alternating current  
 309 (AC) to the skin to measure galvanic skin response (GSR), with a common node of  $V_{TMS}$  for applying  
 310 an AC driving signal (Fig. 4B and C;  $R_a$ ,  $R_b$  and  $R_c$  for 100 k $\Omega$ , 300 k $\Omega$  and 10 k $\Omega$ , respectively).  
 311 The digital readout system compares measured resistances to known reference resistors,  $R_a$ ,  $R_b$   
 312 and  $R_c$  to allow for evaluating sweat rate, GSR and sweat conductivity, respectively (Fig. 4C). The  
 313 analog to digital converter (ADC) ports on the NFC microcontroller (RF430, ISO15693 interface)  
 314 acquire data from the three electrodes of  $R_L$ ,  $R_G$  and  $R_R$  (Fig. 4C). The ADC output voltages for  
 315 each channel can be described by the following equations:

$$316 \quad \text{ADC0} = V_{TMS} \times \frac{R_a}{R_a + R_L} - V_f, \text{ADC1} = V_{TMS} \times \frac{R_b}{R_b + R_G} - V_f, \text{ADC2} = V_{TMS} \times \frac{R_c}{R_c + R_R} - V_f \quad (\text{Eq. 3})$$

317 where  $R_L$  is the resistance across the pair of electrodes in main channel,  $R_G$  is the resistance across  
 318 the electrodes for GSR,  $R_R$  is the resistance at the reference electrode and  $V_f$  is the forward voltage  
 319 of the rectifier ( $\sim$ 150 mV) (16). Fig. 4D shows the terminals for GSR (left; *SI Appendix*, Fig. S13E)  
 320 and the tracking and reference electrodes that couple with the embedded electrodes in microfluidic  
 321 channel (right). The acquired data passes wirelessly to the smartphone. *SI Appendix*, Fig. S13F  
 322 provides details on the individual terminals of the RF430 and TSV632 and the layer of PDMS that  
 323 prevents ingress of external moisture, respectively.

324 *SI Appendix*, Fig. S14A summarizes conductance measurements from ADC0 for artificial  
 325 sweat collected in the main microchannel for various electrolyte concentrations. The captured  
 326 sweat volume depends linearly on sweat filling length along the channel (*SI Appendix*, Fig. S6A)  
 327 and, as a result, on the resistance at the tracking electrodes according to:

$$328 \quad L = \alpha \cdot R_R / R_L \quad (\text{Eq. 4})$$

329 where  $L$  is the filling length ( $L = 0$ -165 mm) and  $\alpha$  is a coefficient that accounts for the ratio of the  
 330 lengths of the reference and tracking electrodes (reference electrode: 1.5 mm; tracking electrodes:  
 331 165 mm). Measurements at 1 kHz minimize the dependence of impedance phase on the  
 332 conductivity of the skin and sweat (16) (*SI Appendix*, Fig. S14B-D). Bench-top and field testing  
 333 with volunteer subjects determine the relationship between ADC2 and sweat conductivity (*SI*  
 334 *Appendix*, Fig. S14E and Fig. 4E for bench-top and field testing, respectively).

335 Evaluations of skin conductivity using the GSR electrodes, as shown in *SI Appendix*, Fig.  
 336 S13E and comparison with sweat rate provide important insights into sweat gland activity, including  
 337 sweat rate and ion reabsorption. Coupling of electronics module with the skeletal microfluidics  
 338 electronically enables sweat rate data collecting. The magnets (3-mm diameter,  $\sim$ 0.5-mm  
 339 thickness), attached to the electronics module, offer robust magnetic forces for mechanical coupling

340 with the electrode terminals (16) (Fig. 1A and *SI Appendix*, Fig. S14F). Previous studies  
341 demonstrate that sweat electrolyte concentrations increase with increasing sweat rate (55-57).  
342 Prolonged exercise on a stationary bike induces high sweat rates, which could give rise to ion  
343 reabsorption. Fig. 4F shows results that correlate  $\Delta$ GSR with skin temperature for the initial phases  
344 of exercise (~10 min at 18°C room temperature). A warmup period of 10-15 min leads to sweating  
345 and development of a stable skin temperature (58). Fig. 4G shows representative  $\Delta$ GSR data  
346 collected from the forearm of a subject. The eccrine glands selectively reabsorb ions, especially  
347 sodium, during sweating as the basis of physiological regulation for homeostasis. The constant  
348  $\Delta$ GSR trend shown in Fig. 4G reflects this type of physiological regulation at low sweat rate phase  
349 of under ~0.8  $\mu$ L/min. Further exercise without hydration induces over-perspiration and perturbs  
350 the regulation system. The  $\Delta$ GSR data is consistent with this behavior, as it increases after 0.7  
351  $\mu$ L/min sweat rate as the ion resorption rate exceeds the excretion rate (59-62) (Fig. 4G).

352

353 **Demonstrations and field testing.** Field tests illustrate capabilities in measurement of cortisol,  
354 glucose and ascorbic acid along with digital tracking of sweat rate and GSR through studies with  
355 four healthy volunteer subjects engaged in physical exercise on a stationary bike in a gym  
356 environment (*SI Appendix*, Fig. S15A-C; 18-22°C temperature and 15-30% humidity). The initial  
357 set of experiments involve data collection from subjects #1 and #2 in the morning and evening.  
358 Sweating was induced within 30 min after the subject woke up at ~7am and before going to sleep  
359 around 7pm (Fig. 5A and B). Intensive work periods ensued for ~7 days (including working  
360 overnight for several days with consumption of caffeine) and rest (regular patterns of sleeping and  
361 meals) for 14 days, serving as short-term stressors. Additional short-term studies with subjects #3  
362 and #4 focused on cortisol, glucose and ascorbic acid measurements along with measurements of  
363 sweat rate and GSR (Fig. 5C-J) in the morning and evening during intensive work, rest with healthy  
364 diet, and control (63-65).

365 *SI Appendix*, Fig. S16 shows these sequences of events and methods for capturing sweat  
366 information at each stage using a smartphone. The filling of sweat into the microchannels activates  
367 the glucose and ascorbic acid assays within ~10-15 min after the start of the stationary bike  
368 exercise. Upon filling of the reservoirs for glucose and ascorbic acid assays, a smartphone camera  
369 with the shielding module (Fig. 3B and *SI Appendix*, Fig. S12F) captures the fluorescent signals.  
370 As sweat fills into the main channel, measurements of sweat rate and GSR (4~13 separate times)  
371 can be performed by placing the smartphone in proximity to the device. The LFIA assay activates  
372 after complete filling of the main channel (~70  $\mu$ L). The digital camera on the smartphone captures  
373 the developed color.

374 Fig. 5A-D compares measurements of cortisol conducted with the LFIA and a bench-top  
375 ELISA protocol in the morning and evening, under intensive work and resting conditions. Under  
376 ordinary, routine circumstances, diurnal variations of cortisol level among the subjects exhibit  
377 standard patterns, with concentrations that peak in the morning, to facilitate focus during the  
378 daytime, and then gradually decrease through the afternoon and evening. This cortisol circadian  
379 rhythm is apparent in data from subjects #3 and #4, as shown in Fig. 5C and D (64, 65). The  
380 subjects, during intensive work, experience physiological stress, fatigue and irregular life patterns  
381 with inadequate sleep. These conditions disrupt the cortisol-melatonin circadian rhythm. As a  
382 result, the hypothalamus region of the brain produces corticotropin releasing hormone (CRH),  
383 which in turn, activates the hypothalamic-pituitary-adrenal axis and stimulates anterior pituitary  
384 activity (23, 24, 66), thereby increasing cortisol production and disrupting of cortisol circadian  
385 rhythm (67). The cortisol levels for Subject #2, #3 and #4 during intensive work show disruptions  
386 of circadian rhythm, consistent with physiological conditions that reflect exhaustion (Fig. 5B and C).  
387 This disruption could aggravate the psychological state (e.g. anxiety, insomnia, etc.). Fig. 5K  
388 demonstrates the accuracy and reliability of the LFIA in the device compared with bench-top ELISA  
389 results ( $R^2=0.7974$ ). The control tests measuring saliva cortisol levels before and after exercise  
390 show that the effect of exercise intensity on cortisol level is small (68) compared to circadian rhythm  
391 changes that occur during the day. After intense work condition, the subjects return to a routine  
392 life pattern for 2 weeks and consume ascorbic acid (1000 mg per a day for subject #3 and #4) (69).  
393 The result is that the cortisol patterns recover to normal circadian rhythm, as shown in Fig. 5A-C.  
394 Although subjects #1-#3 show lower cortisol levels after 14 days, the relative changes in sweat  
395 cortisol levels due to circadian rhythm appear to dominate compared to dietary interventions for  
396 subject #4 (Fig. 5D). Fig. 5E and F show that the ascorbic acid levels increase from  $\sim 0 \mu\text{M}$  to  $\sim 0.33$   
397  $\mu\text{M}$  for subject #3 and from  $\sim 0 \mu\text{M}$  to  $\sim 0.42 \mu\text{M}$  for subject #4, as a result of vitamin C intake. By  
398 contrast, the glucose levels exhibit no specific trends, i.e.  $\sim 0.62 \mu\text{M}$  and  $\sim 0.80 \mu\text{M}$  for subject #3  
399 and  $0.84 \mu\text{M}$  and  $0.81 \mu\text{M}$  for subject #4, as mean values of measurements taken on day 0 and  
400 day 14. *SI Appendix*, Fig. S15D and E show glucose and ascorbic acid measurements at day 0,  
401 2, 6, 10 and 14 for these same two subjects. These results show that the device along with  
402 integrated assays have practical utility, as the glucose and ascorbic acid sensitivity ranges are  
403 within previously reported physiological ranges (3, 70).

404 Representative results for sweat rate and  $\Delta\text{GSR}$  appear in Fig. 5I-J. These findings  
405 establish correlations between  $\Delta\text{GSR}$  and sweat rate, likely associated with resorption and  
406 secretion of ions due to sweating. Sweat rate measurements from Subjects #1 and #2 appear in  
407 Fig. 5H and I, with comparisons to  $\Delta\text{GSR}$  in *SI Appendix*, Fig. S15F and G. Wirelessly acquired  
408 data from ADC0 and ADC2 yield the sweat rate and electrolyte concentrations, respectively.  
409 Comparisons of  $\Delta\text{GSR}$  and sweat rate in Fig. 5I and J suggest that resorption behavior occurs for

410 secreted ions (i.e. sodium) from the proximal secretory coil (18, 59, 70-72). In the limiting case,  
411 the rate of secretion of ions exceeds the rate of resorption, thereby leading to an increase in  $\Delta$ GSR  
412 at a critical sweat rate (73-74). Fig. 5I and J shows that  $\Delta$ GSR measurements for subjects #3 and  
413 #4 remain steady until the sweat rate reaches  $\sim 1.1 \mu\text{L}/\text{min}$  for subject#3 and  $\sim 0.8 \mu\text{L}/\text{min}$  for subject  
414 #4, at which point the  $\Delta$ GSR increases.

## 415 **Conclusion**

416 Eccrine sweat is an interesting, yet incompletely understood, class of biofluid that contains  
417 a range of chemical species whose concentrations could provide significant information on  
418 physiological status. The potential relevance spans sports science, diagnosis in clinical medicine  
419 and evaluations of military readiness. The multifunctional device platform described here exploits  
420 a soft microfluidic network of hard channels and reservoirs, with integrated flexible electronic  
421 systems, as a practical lab-on-a-chip type system with immunoassays, fluorometric detection  
422 capabilities and wireless functionality tailored specifically to monitoring of physical and mental  
423 stresses. A key feature is the lateral flow integration for immunoassay analysis of sweat cortisol.  
424 The fluorescence assay provides information on other trace substrates such as glucose and  
425 ascorbic acid. Wireless modes of operation based on NFC protocols support real-time, wireless  
426 digital tracking of sweat rate and GSR. Field tests on human subjects engaged in activities to  
427 create and then relieve stresses demonstrate the utility of the technology in this important context,  
428 as well as its ability to address scenarios of practical interest. The versatile design principles  
429 introduced here can be configured to address many additional capabilities in sweat collection,  
430 storage and chemical analysis in remote field settings (e.g. modified skin mounted microfluidics, in  
431 which micro-photodetector and excitation light are embedded to enable FL readouts).  
432  
433

434

## 435 **Materials and Methods**

436 **Fabrication of soft skeletal microfluidics with flexible electrodes system.** Fabrication began  
437 with the formation of a mold from a silicon wafer patterned using photolithography techniques.  
438 More precisely, photoresist KMPR1010 was spin-cast on a silicon wafer at 3000 rpm for 30 s, baked  
439 on a hot plate at  $110^\circ\text{C}$  for 3 min, exposed to UV irradiance at  $300 \text{ mJ}\cdot\text{cm}^{-2}$  for 2 min and developed  
440 with developer MF917. Deep reactive-ion etching (polymethylmethacrylate coating; STS Pegasus  
441 ICP-DRIE, SPTS Technologies Ltd, UK) removed the exposed silicon to a selected depth ( $\sim 400$   
442  $\mu\text{m}$ ). A prepolymer to polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, Midland, MI, mixed  
443 at a 10:1 ratio of base to curing agent by weight) was then cast and cured on the silicon structures  
444 to yield soft molds. These molds were used to form structures of NOA (Norland Optical Adhesive;  
445

446 NOA 81, Norland Products, Inc., Cranbury, NJ; Partial curing; Expose to 30 W UV light for 4~10  
447 min; *SI Appendix*, Fig. S1B).

448 Fabrication of Cu electrodes relied on a photolithographic process, whereby photoresist  
449 (AZ4620) was spin-cast at 3000 rpm for 30 s, and then baked on a hot plate at 60°C for 1 min.  
450 After UV irradiance at 300 mJ·cm<sup>-2</sup> to expose the photoresist, a development process for 1 min  
451 yielded the desired pattern. Next, wet etching with copper etchant (HFCE100, Transense,  
452 Oxfordshire, UK) of Cu foils (DuPont, Wilmington, DE) laminated onto glass slides (Fisherbrand,  
453 Pittsburgh) coated with PDMS (Sylgard 184, Dow Corning, Midland, MI; mixed at a 20:1 ratio of  
454 base to curing agent by weight and partially cured on a hot plate at 110°C for 3 min; *SI Appendix*,  
455 Fig. S3A) removed the exposed regions of the Cu. Casting a ~500 µm thick layer of NOA 81 on the  
456 patterned Cu-PDMS substrate and exposing to UV light (30 W for 4 min) enabled transfer of the  
457 Cu electrodes to the surface of the NOA 81 (*SI Appendix*, Fig. S3A). Assembly of NOA microfluidic  
458 trenches (*SI Appendix*, Fig. S1D) and electrodes (*SI Appendix*, Fig. S3B) exploited uncured NOA81  
459 to create sealed channels with precise alignment (*SI Appendix*, Fig. S3C). A laser cutter  
460 (ProtoLaser R; LPKR, Germany) defined the perimeter of the assembly as the final step to complete  
461 the fabrication. *SI Note 4*

462

463 **Lateral flow immunoassay platform preparation.** The addition of 1 M NaOH to colloidal 30-nm  
464 gold nanoparticles (GNP; Sigma-Aldrich, St. Louis, MO) shifted the pH close to 7.0. Adding 0.5  
465 mg/mL anti-cortisol antibody (ACA; ab1951, Abcam Inc, Cambridge, UK) to a final concentration of  
466 ~0.5 µg/mL and incubating (rotating at 30 rpm) the solution for 1, 3, 20 min enabled spontaneous  
467 conjugation of antibody onto the activated GNP. Adding 10% (w/v) Bovine serum albumin (BSA;  
468 to final concentration of 0.1%; Sigma, St. Louis, MO), allowing stabilization at room temperature for  
469 1 h, centrifuging (9000 g for 30 min at 4 °C, followed by ~4 times repetition of washing-resuspending  
470 of precipitated pellet with a storage buffer; PBS buffer includes 1% BSA and 2% sucrose) and  
471 drying of the separated precipitation at room temperature for 4 h yielded anti-cortisol antibody  
472 conjugated gold nanoparticles (ACA-GNP; stored at 4°C).

473 Cortisol-BSA and IgG antibody were immobilized on a nitrocellulose membrane (pore size:  
474 5, 8, 10, 12, 15 µm; Advanced Microdevices, Ambala Cantt, India) as the control and test lines,  
475 respectively (Claremont BioSolutions, LLC, CA). A sample-conjugation pad (Advanced  
476 Microdevices, Ambala Cantt, India) was saturated with ACA-GNP for 1 h and then dried for 30 min  
477 at 37 °C. The prepared nitrocellulose membrane and an absorbent pad (Filter paper No.1,  
478 Whatman, GE Healthcare Life Sciences, UK), as shown in *SI Appendix*, Fig. S9C, served as  
479 supports for the lateral flow immunoassay. Standard protocols for ACA, Cortisol-BSA, IgG antibody  
480 and BSA set a 1-year shelf life from when the package is delivered.

481

482 **Electronics design and assembly.** Fabrication began with patterning of a two-layer printed circuit  
483 board by processing of multilayer foils of Cu-PI-Cu (18  $\mu\text{m}$ /75  $\mu\text{m}$ /18  $\mu\text{m}$ ) with a UV laser cutter  
484 (ProtoLaser U4; LPKF, Germany). The main processor, RF430FRL152HCRGER (RF430, ISO/IEC  
485 15693, ISO/IEC 18000-3; Texas Instruments, Dallas, TX) served as the NFC platform, with the  
486 ability to rectify incident power from a smartphone device at up to 720  $\mu\text{W}$  at 2.0 V, depending on  
487 coupling efficiency, and relaying data over the 13.56 MHz communications link. The RF430  
488 supports 14-bit Sigma-Delta ADC with triple analog inputs at an input range up to 900 mV and  
489 maximum sampling frequency of 2 kHz, down-sampled to 1 Hz resolution. Signal amplification and  
490 measurement of the main and reference channels used another chip, TSV634QFN16  
491 (STMicroelectronics, Geneva, Switzerland), as a four-channel operation amplifier with low energy  
492 consumption (60  $\mu\text{A}$  at 5V) and large unity gain-bandwidth (800 kHz). On power-up, the system  
493 sourced a 5 kHz, rail-to-rail square wave to the channels, causing an AC current to flow through  
494 the collected sweat. The magnitude of this current is proportional to the concentration of ions in  
495 sweat, as an electrical impedance that causes the sourced 5 kHz waveform to attenuate during  
496 passage through the sweat. This attenuation reduces the amplitude of the waveform whose  
497 rectified voltage is buffered and measured by the TSV634 and ADC, respectively. This rectified  
498 voltage level is sampled, processed, and relayed to an NFC compatible reader by the RF430. An  
499 NFC-compatible smartphone (LG Nexus 5X; LG, Seoul, South Korea) and custom-developed  
500 application for the Android operating system enabled visualization of the data.

501

502 **Field studies.** Field studies with four healthy volunteers who exercise regularly involved operating  
503 stationary bike in gym environment (18-20°C temperature and 15-30% humidity) before, during and  
504 after intensive works. The intensive works involve mostly the environment of mental stress (i.e.  
505 long studying or research time with irregular sleeping pattern) that is followed by ascorbic acid  
506 supplement of 1,000 mg/day for the next 14 days (75). All subjects provided signed consent, and  
507 had medical consultations before and after field tests with a medical doctor. This study was  
508 approved by the Institutional Review Board (IRB: STU00208494) at Northwestern University.  
509 Control tests using saliva from the subjects verified circadian rhythm changes observed in sweat  
510 cortisol levels. Prior to mounting the devices, the skin was cleaned with 70% ethanol. Subjects  
511 wore sportswear (shorts and t-shirt) for the tests.

512

### 513 **Acknowledgments**

514

515 J.K. acknowledges the support from National Research Foundation of Korea (NRF-  
516 2020R1F1A1068083). R.G., A.J.A., and J.B.M. acknowledge funding support from the Leo Science



517 & Tech Hub. S.H.S. acknowledges the support from the Primary Research Program (20A01021)  
518 of the Korea Electrotechnology Research Institute. This work utilized Northwestern University  
519 Micro/Nano Fabrication Facility (NUFAB), which was partially supported by Soft and Hybrid  
520 Nanotechnology Experimental (SHyNE) Resource (NSF ECCS-1542205).

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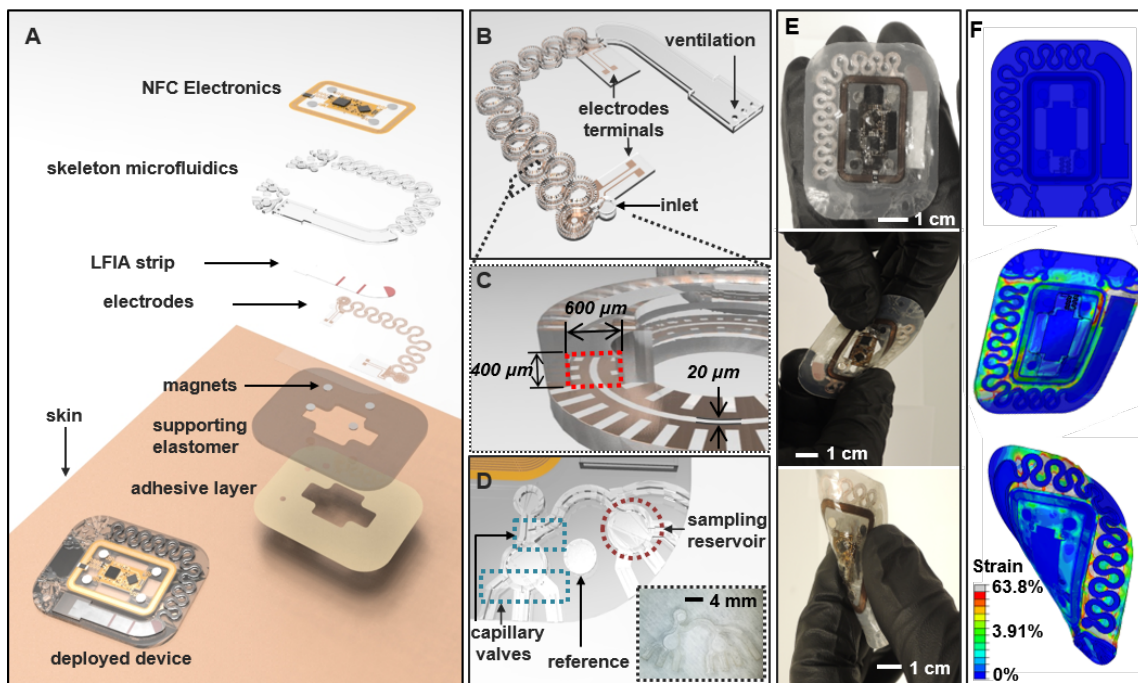
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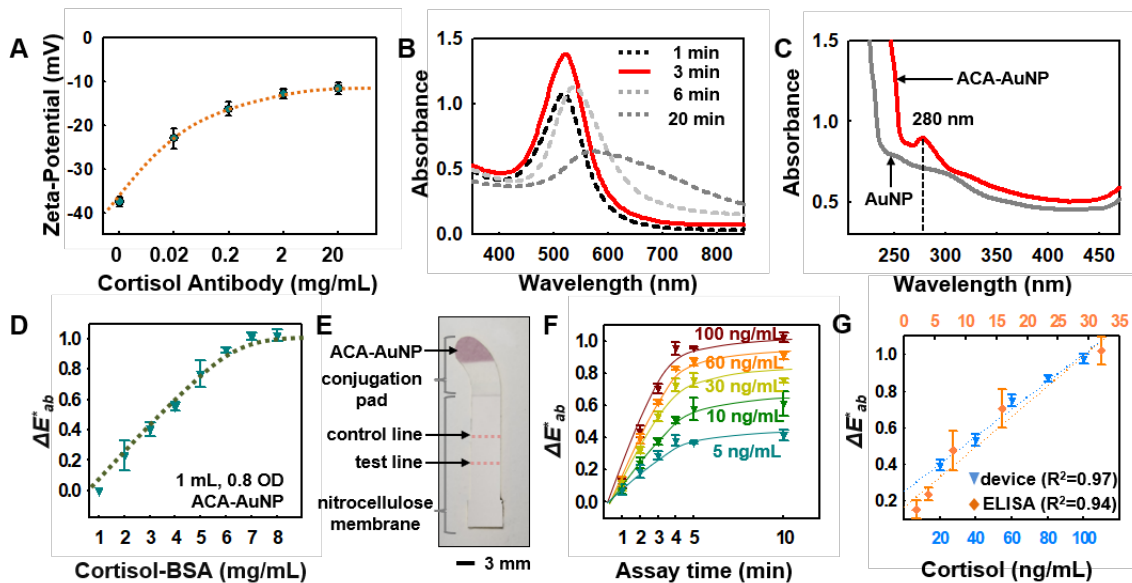
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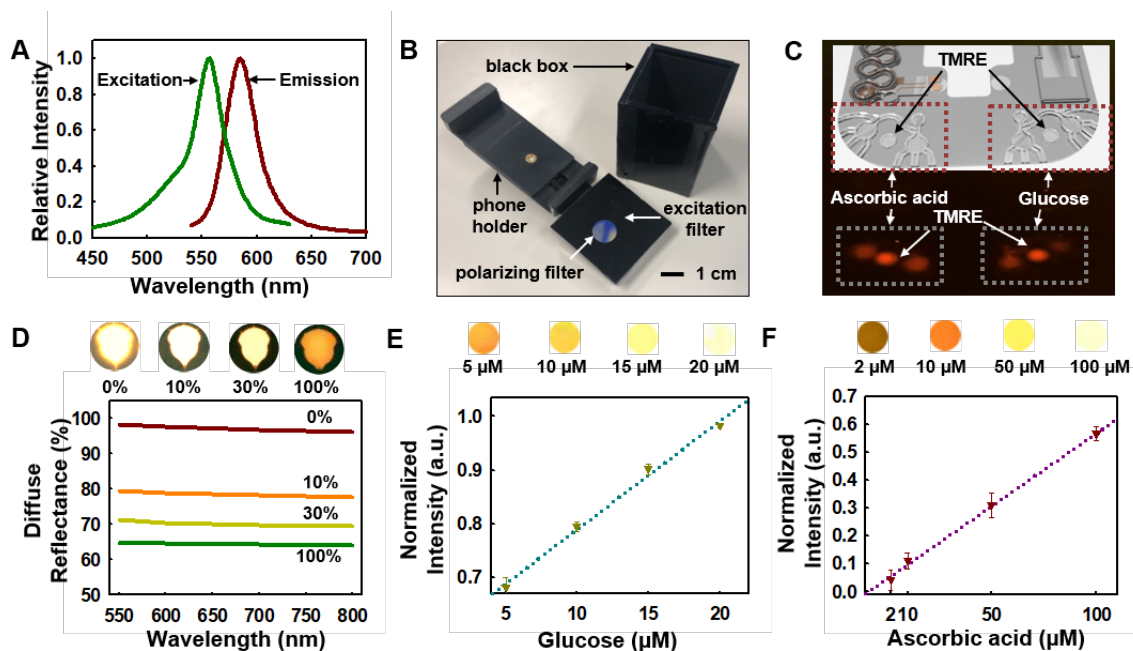
695 **Fig. 1** Schematic illustrations and optical images of a skeletal microfluidic device for immunoassays  
 696 for cortisol, fluorescence assays for glucose and ascorbic acid (vitamin C) and electrical interfaces  
 697 for sweat loss, sweat rate and galvanic skin response. (A) Exploded schematic illustration of the  
 698 structure of the device. (B) Magnified view of the main serpentine skeletal channel for tracking  
 699 sweat loss, sweat rate, and an immunoassay for cortisol. (C) Cross-sectional view of the main  
 700 channel, highlighting channel dimensions and integrated electrodes. (D) Microfluidic structures for  
 701 fluorescence assays of glucose and ascorbic acid and an optical image of the system (inset). (E)  
 702 Optical image of an assembled device (top), and undergoing mechanical twisting (middle) and  
 703 bending (bottom). (F) 3D modeling of the mechanics associated with similar configurations: flat  
 704 (undeformed; top), twisted (middle) and bent (bottom) to show the corresponding distributions of  
 705 strain.



706

707 **Fig. 2** Immunoassay-based lateral flow design and measurements for sweat cortisol. (A) Zeta  
 708 potential values measured after conjugation of ACA (0, 0.02, 0.2, 2 and 20 mg/mL; 3 data points  
 709 for each ACA concentration; N=15) on 30-nm AuNPs. (B) Effects of ACA (0.5 mg/mL) conjugation  
 710 time on absorbance. (C) Comparison of absorbance at a wavelength of ~280 nm before and after  
 711 ACA conjugation. (D) Color development of ACA-AuNP at various concentrations of cortisol-BSA  
 712 on the NC membrane. (E) Optical image of the lateral flow immunoassay strip after assembly and  
 713 laser cutting. (F) Color development trends at various cortisol concentrations (5, 10, 30, 60 and  
 714 100 ng/mL) as a function of time. (G) Calibration of color index from the device at various  
 715 concentrations of cortisol (20, 40, 60, 80 and 100 ng/mL) and comparisons to bench-top ELISA  
 716 tests at concentrations of 2, 4, 8, 16 and 32 ng/mL.

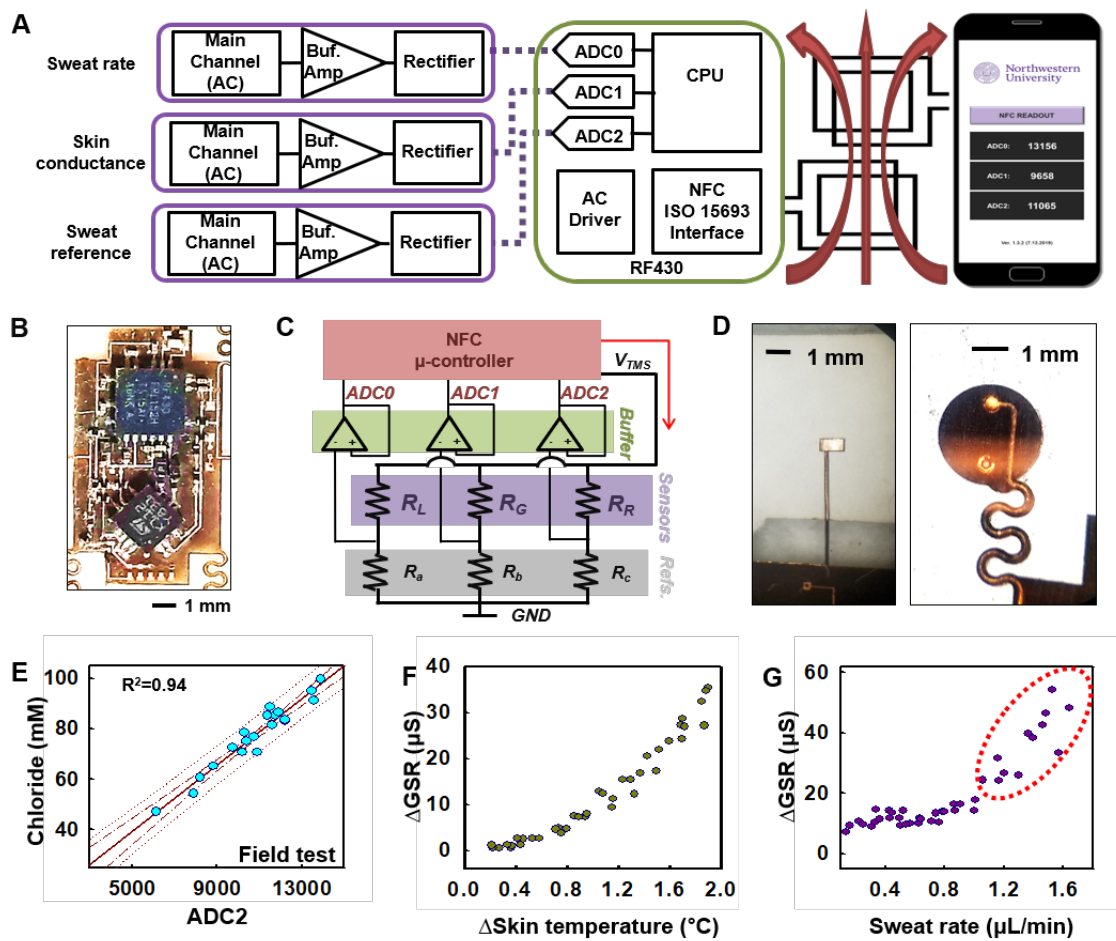
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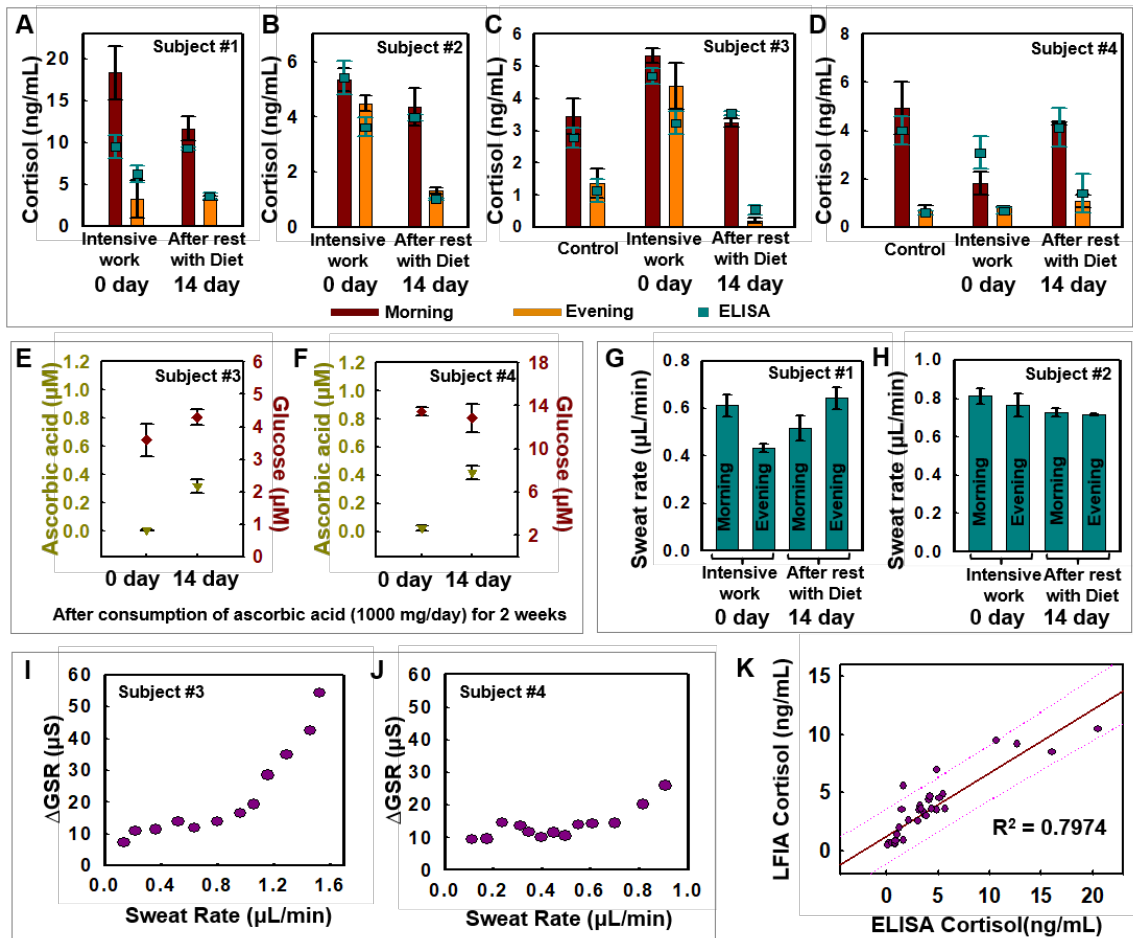
719 **Fig. 3** Fluorescence assay design and measurements for sweat glucose and ascorbic acid. (A)  
 720 Excitation and emission curves of OxiRed, the fluorescence probe. (B) Optical image of the  
 721 apparatus used for fluorescence readout. (C) Image of ascorbic and glucose signals along with  
 722 the reference (TMRE) signal. (D) Effect of the silicone packaging on the fluorescent signal for  
 723 various ratios at black and white pigments at 0:10, 1:9, 3:7 and 10:0 (0, 10, 30 and 100%,  
 724 respectively), along with corresponding images (top). (E) Plot of the normalized fluorescence  
 725 intensity for various glucose concentrations at 0.1, 0.5, 1, 2  $\mu\text{M}$  and their fluorescence intensities  
 726 from associated images (top). (F) Plotting of normalized fluorescence intensity for various ascorbic  
 727 acid concentrations at 5, 10, 50, 100  $\mu\text{M}$  concentrations and their fluorescence intensities from  
 728 associated images (top).





729

730 **Fig. 4** Design of NFC electronics for monitoring sweat loss, sweat rate, and GSR. (A) Schematic  
 731 block diagram of the NFC electronic system and its interface to a sweat microfluidic device and a  
 732 smartphone. (B) Optical image of the electronics to show chip placement. (C) Schematic block  
 733 diagram of the electronics to show the reference resistor layouts for the main, reference, and GSR  
 734 readout. (D) Magnified optical images of the electrode terminals for GSR (left) and tracking-  
 735 reference to the microfluidic device (right). (E) Plot of electrolyte concentration for a series of  
 736 samples of human sweat in the reference microchannel and corresponding ADC2 values  
 737 determined by wireless readout. (F) Effect of body temperature at the initial phase of exercise on  
 738  $\Delta$ GSR. (G) Correlation between sweat rate and  $\Delta$ GSR after skin temperature stabilizes and  
 739 sweating begins (forearm, 18-20 $^{\circ}$ C temperature, and 15-30% humidity).



740

741 **Fig. 5** On-body measurements of sweat biomarkers during exercise. (A-D) Cortisol lateral flow  
 742 immunoassay results for subjects #1 and #4 at 0 and 14 days. 'Control' (C, D) indicates  
 743 measurements of sweat cortisol under normal conditions of that the subjects are not stressed. (E,  
 744 F) Results of ascorbic acid and glucose at 0 and 14 days for subjects #3 and #4. (G, H) Sweat rate  
 745 measurements for subjects #1 and #2 at 0 and 14 days. (I, J)  $\Delta$ GSR measurements during high  
 746 intensity exercise and sweating for subjects #3 and #4. (K) Plotting and regression of quantitative  
 747 assays results from LFIA and ELISA. Dotted line is prediction line.