Electronic Supplementary Material (ESI) for Analyst

Screening Reactive Metabolites Bioactivated by Multiple Enzyme Pathways Using a Multiplexed Microfluidic System

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Supporting Information:

Chemicals.

2-naphthylamine (2-NA, M_W=143.19), 2-aminofluorene(2-AF, M_w=181.23), 4-aminobiphenyl (4-ABP, M_W=169.22), *N*-(9*H*-fluoren-2-yl)acetamide (2-AAF, M_w=223.27), tetrahydrofuran (THF, M_W=72.11), poly(diallyldimethylammonium chloride) (PDDA, average M_w=100,000-200,000), poly(sodium 4-styrenesulfonate) (PSS, average M_W=70000), calf thymus DNA (Type I), acetyl coenzyme A sodium salt (AcCoA), D,L-dithiothreitol (DTT), ethylenediaminetetraacetic acid disodium salt (EDTA), carnitine acetyltransferase from pigeon breast muscle (CAT, EC 2.3.1.7), acetyl-D,L-carnitine hydrochlodride(ACH) and all other chemicals were from Sigma. Pooled male human liver microsomes (HLM, 20 mg mL^{-1} in 250 mM sucrose) were from BD Gentest (Woburn, MA) and contained (a) 20 mg mL^{-1} total protein content, (b) total cyt P450 content of 340 pmol mg^{-1} of protein using the method of Omura and Sato,[1] (c) CPR activity of 230 nmol cyt c reduced (mg of total protein × min)$^{-1}$, (d) 550 pmol mg^{-1} cyt b_5. Specific cyt P450 enzyme activities in picomole product per mg of total protein per minute were: (a) cyt P450 3A–3200 from testosterone 6β-hydroxylase assay, (b) cyt P450 2C9–2500 from diclofenac 4'-hydroxylase assay, (c) cyt P450 2E1–2200 from chlorzoxazone 6-hydroxylase assay, (d) cyt P450 1A2 – 750 from 7- ethoxyresorufin O-diethylase assay, and (e) cyt P450 4A–1700 from [^{14}C] lauric acid-hydroxylase assay. Human NAT 1 cytosol from BD Gentest (Woburn, MA) and contained 2.5 mg mL^{-1} total protein content in 20 mM Tris-HCl (pH=7.5), 1.0 mM EDTA, 1.0 mM DTT. Human microsomal epoxide hydrolase (EH), 10 mg/mL in 100 mM potassium phosphate buffer of pH 7.4, pooled human liver S9 (Hs9) 20.0 mg/mL in 150 mM KCl, 50 mM Tris-HCl +2.0 mM EDTA; and baculovirus-insect cell expressed cyt P450 1B1supersomes (cyt 1B1), 4.5 mg/ml in 100mM potassium phosphate buffer of pH 7.4 were from BD Gentest (Woburn, MA). The bicinchoninic acid (BCA) protein assay kit (μBCA assay kit, model number = 23235) was from Thermoscientific (IL, USA).

Microfluidic set up fabrication.

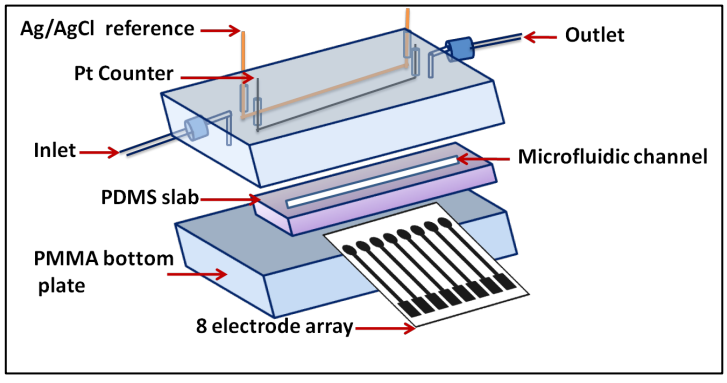

Fig. S1. A three dimesional view of a single microfludic set up.

The poly(dimethyl)siloxane (PDMS) kit (Dow Corning) was used to make PDMS microfluidic channel. A 10:1 mixture of the base and curing agent was mixed well and poured into an aluminum mold. The mixture was cured at 80°C for 2 hours forming a microfluidic channel that is 1.5 mm wide, 2.8 cm long, with 63 µL volume. The flexible PDMS channel was placed on top of the screen printed carbon electrode array (Kanichi Inc.) and was sandwiched between two hard poly(methylmethacrylate) (PMMA) plates bolted together with screws (Fig. S1) to form a sealed channel. Ag/AgCl reference and Pt counter electrode wires were placed along the 8 sensor electrodes to minimize cross talking, which were given access to the outside via two holes in the top PMMA plate. The top PMMA plate was equipped with 4 mm diameter female ports to connect to 0.2 mm i.d. polyether ether ketone (PEEK) tubing forming an inlet and an outlet.

Four of such microfluidic devices were connected in parallel fashion and buffer solutions were driven through them by a syringe pump (Harvard apparatus Inc., 55-3333) connected to an injector valve (Rheodyne, 9725i) and switching valve (Rheodyne, 9060) were used to inject solutions into the microfluidic device via 0.2 mm i.d. PEEK tubing as shown in Fig. S1.

Metabolite generation and measurements.

The PDMS microfluidic channel was placed on the eight electrode array, where PDDA/PSS/$(RuPVP/DNA)_2$/RuPVP/HLM/PDDA/DNA and $(RuPVP/DNA)_2$/RuPVP/HLM/NAT/DNA films were already constructed. This assembly was supported by PMMA plates machined to fit on either side of the PDMS slab, and bolted together tightly to provide a sealed microfluidic channel. Four of such devices were assembled and connected to the pumping system as shown in Fig. S2.The oxygenated substrate containing necessary cofactors for NAT activity, 0.25 mM 2-AAF, 0.2 mM 2-AF, 0.2 mM 2-NA, and 0.05 mM 4-ABP, was injected via the injector valve at a flow rate of 50 $\mu Lmin^{-1}$ where a running buffer of oxygenated 10 mM acetate buffer of pH 5.8 was employed. The selector valve was used to direct the each substrate in to four fluidic channels (See Fig. S2). Metabolite generation via constant potential electrolysis at -0.65 V vs. Ag/AgCl (0.14 M NaCl) was performed by using an eight-electrode CH Instruments 1040A electrochemical workstation coupled with CHI 685 electrochemical multiplexer connected to the microfluidic array system at a substrate flow rate of 50 $\mu Lmin^{-1}$ for a given time interval in one array at a time. Excess substrate remaining inside the channel and tubings was washed away by a constant flow of running buffer at 50 $\mu Lmin^{-1}$ for 5 min. Square wave voltammograms were acquired in anaerobic 10 mM acetate buffer of pH 5.8 under stagnant conditions using CH Instruments 1040A electrochemical workstation coupled with CHI 685 electrochemical multiplexer.

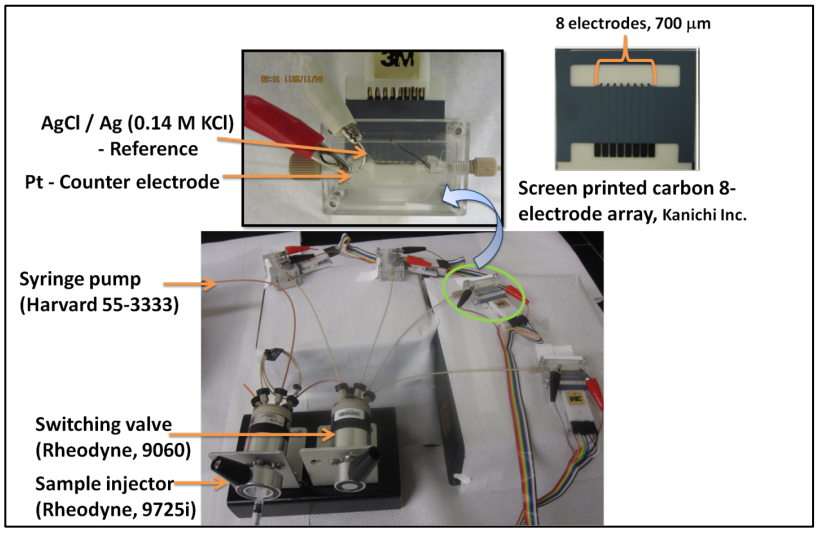

Fig. S2. Microfluidic set up with four microfluidic devices connected in a parallel fashion and the top inset shows a zoomed image of one device with a carbon ink printed 8 electrode array.

Results

Film characterization.

A quartz crystal microbalance (QCM, USI Japan) was utilized to monitor the LbL assembly of the film on 9 MHz QCM resonators (AT-cut, International Crystal Mfg.), where a negatively charged monolayer was constructed on gold (0.16(±0.01) cm^2) by incubating the resonators in 0.5 mM 3-Mercaptopropionic acid in ethanol overnight. The adsorption conditions and stability of each layer was optimized and frequency change (ΔF) was measured after washing with deionized water (D.I. water) and drying over a stream of nitrogen. The mass per unit area M/A (g cm^{-2}) in each layer is related to ΔF, which is given by[2]

$$M / A = -\Delta F(Hz)/(1.83\times10^{8}) \qquad \textbf{(Equation S1)}$$

Where A is the area of the gold disk on the quartz resonator in cm^2. Similarly nominal film thickness, d (nm) is given by[2]

$$d = -(0.016 \pm 0.002)\Delta F(Hz) \qquad \textbf{(Equation S2)}$$

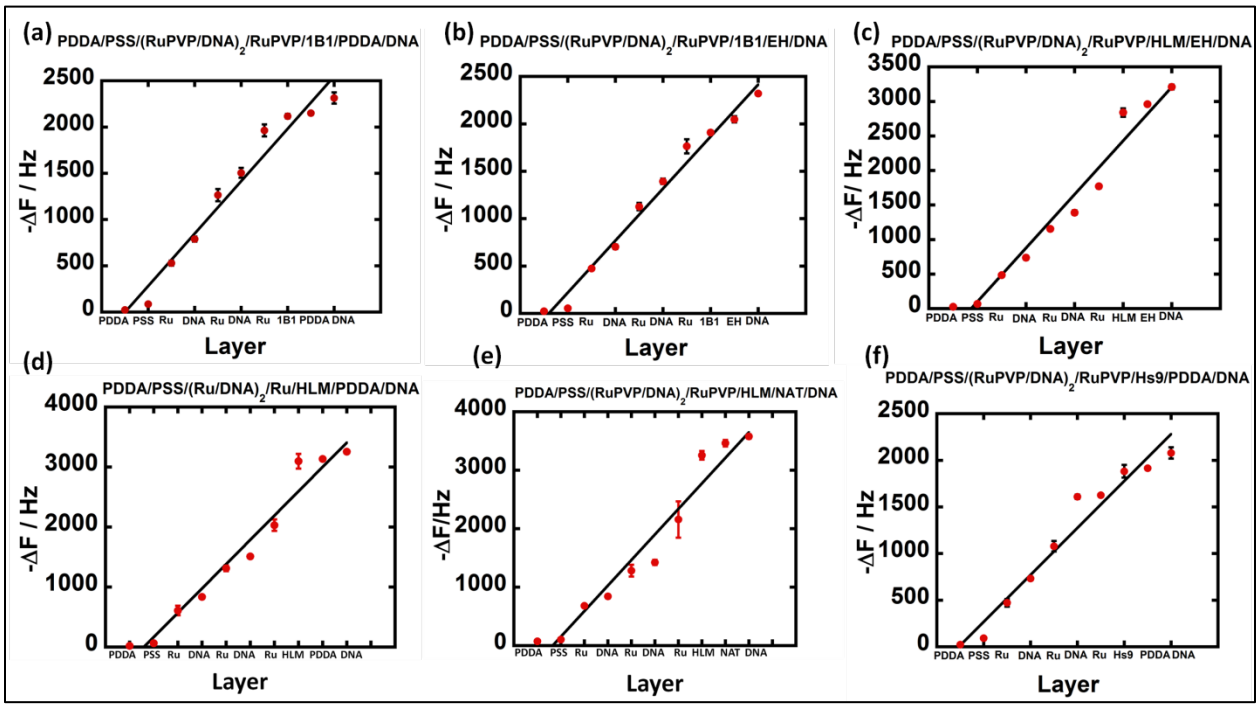

Fig. S3. QCM plots of the films (a) cyt 1B1. (b) cyt 1B1+EH. (c) HLM+EH. (d) HLM. (e) HLM+NAT. (f) Hs9.

The total amount of protein in unit mass of HLM was estimated by standard BCA assay[3] where, a commercial μBCA assay kit (Thermoscientific Inc.) was used. A standard calibration plot (Fig. S4) was constructed by using bovin serum albumin (BSA). A concentration series of BSA :

μBCA reagent (1:1 by volume) was allowed 1 hour to react at 60 °C in a water bath, and the absorbance at 562 nm was measured.

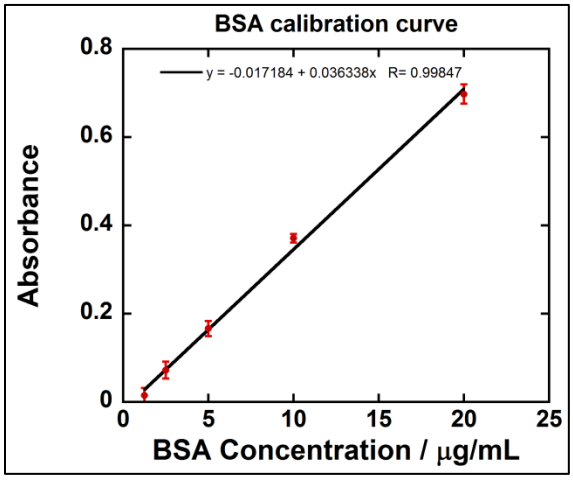

Fig. S4. BSA calibration plot for total protein assay. Absorbance at 562 nm corresponds to each BSA concetration. (Error bars reflect SD for triplicates.)

1 mg of HLM with a volume ratio of HLM: μBCA reagent (1:1) was allowed 1 hour to react at 60 °C in a water bath, and the absorbance at 562 nm was measured. Similarly, protocol was applied to 1 mg of the NAT cytosolic fraction, Hs9 fractions, EH and cyt 1B1 supersomes as well.Thus, from Fig. S4 amount of total protein in 1 mg of each enzyme source was calculated according to the average ratio of protein/BSA is given as1.05 (±0.12) (Thermoscientific Inc.).

A CH instrument 660A electrochemical analyzer was employed to obtain cyclic voltammograms (CV) of the LbL film constructed on an ordinary basal plane pyrolytic graphite (PG, Advanced Ceramics) electrode (A=0.2 cm^2), with an electrochemical cell comprised of a saturated calomel electrode (SCE) and a Pt-wire counter electrode in 50 mM phosphate plus 0.1 M NaCl buffer of pH 7.4 which was extensively purged with purified nitrogen gas at ambient temperature 22.0(±2.0) °C.

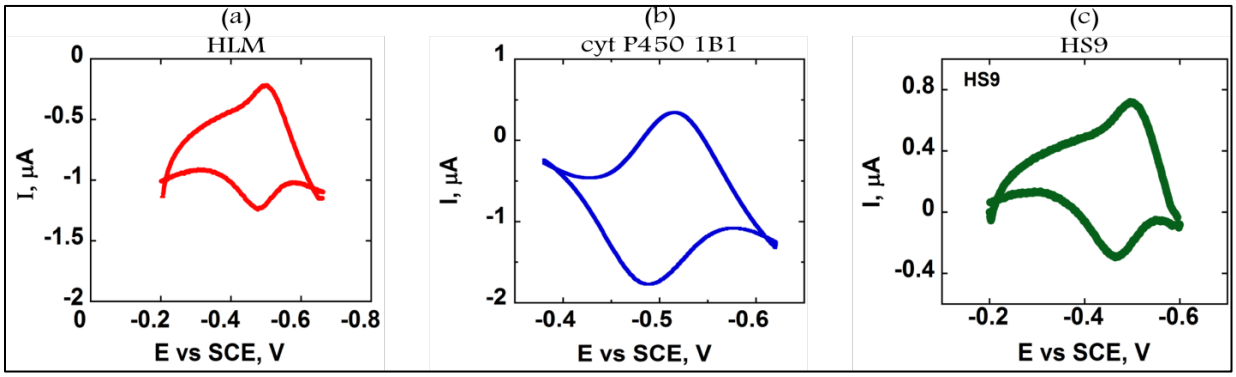

Fig. S5. Background subtracted cyclic voltammograms of (a) HLM (b) 1B1 (c) Hs9 films on a PG electrode in 50mM phosphate + 0.1M NaCl buffer at pH = 7.4 purged with nitrogen at a scan rate of 0.1 Vs^{-1}

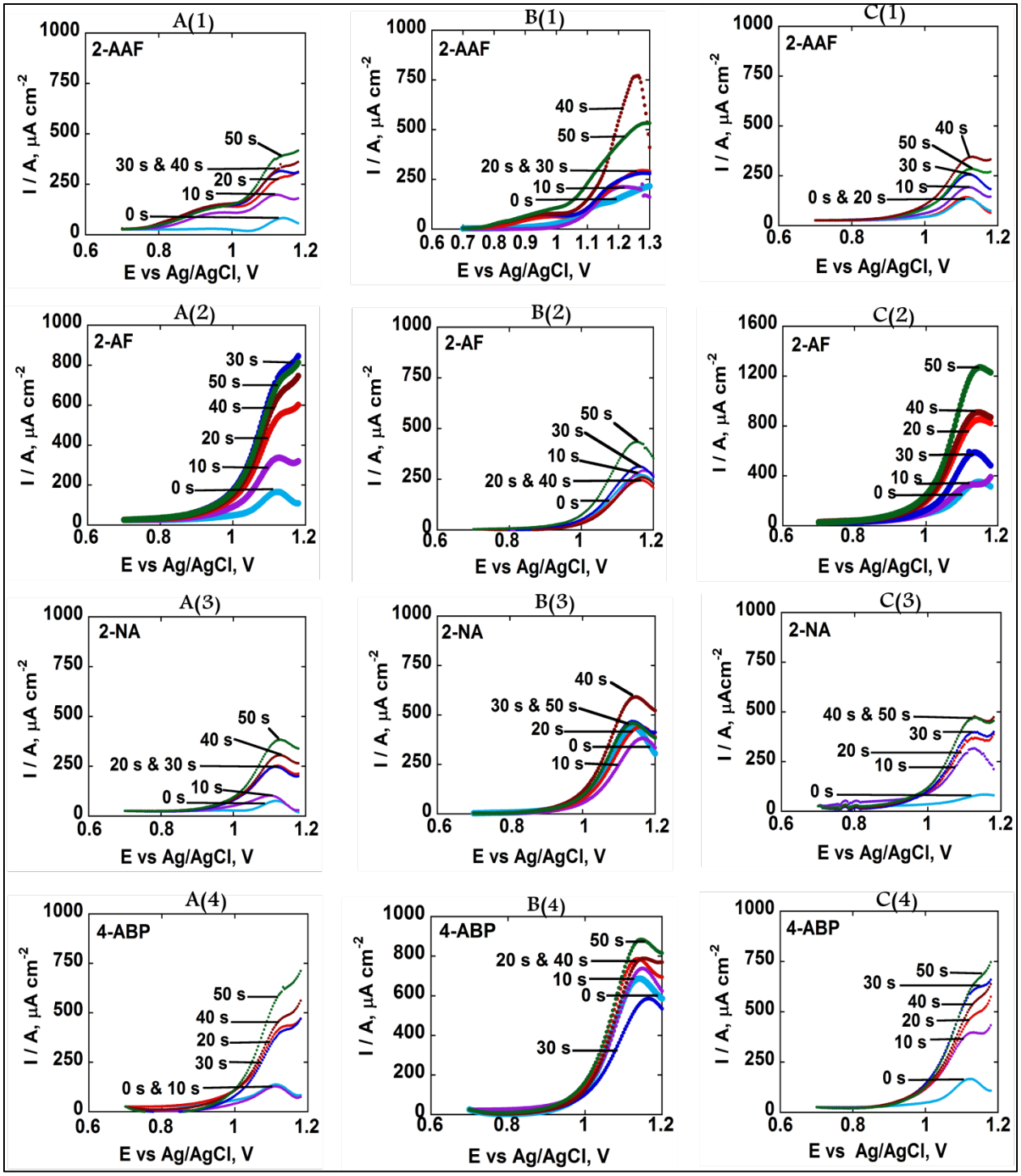

Fig. S6. Background subtracted average difference SWV peak current densities measured sequentially at different enzyme reaction times in anaerobic pH = 7.4 phosphate buffer for multiplexed array sensors featuring optimized films of (A) HLM, (B)HLM+NAT, (C) Hs9, (SWV ampl. 25 mV; freq. 15 Hz; step 4 mV) for the exposure of (1) 0.25 mM 2-AAF, (2) 0.2 mM 2-AF, (2) 0.2 mM 2-NA, (4) 0.05 mM 4-ABP/0.01 mM 4-ABP.

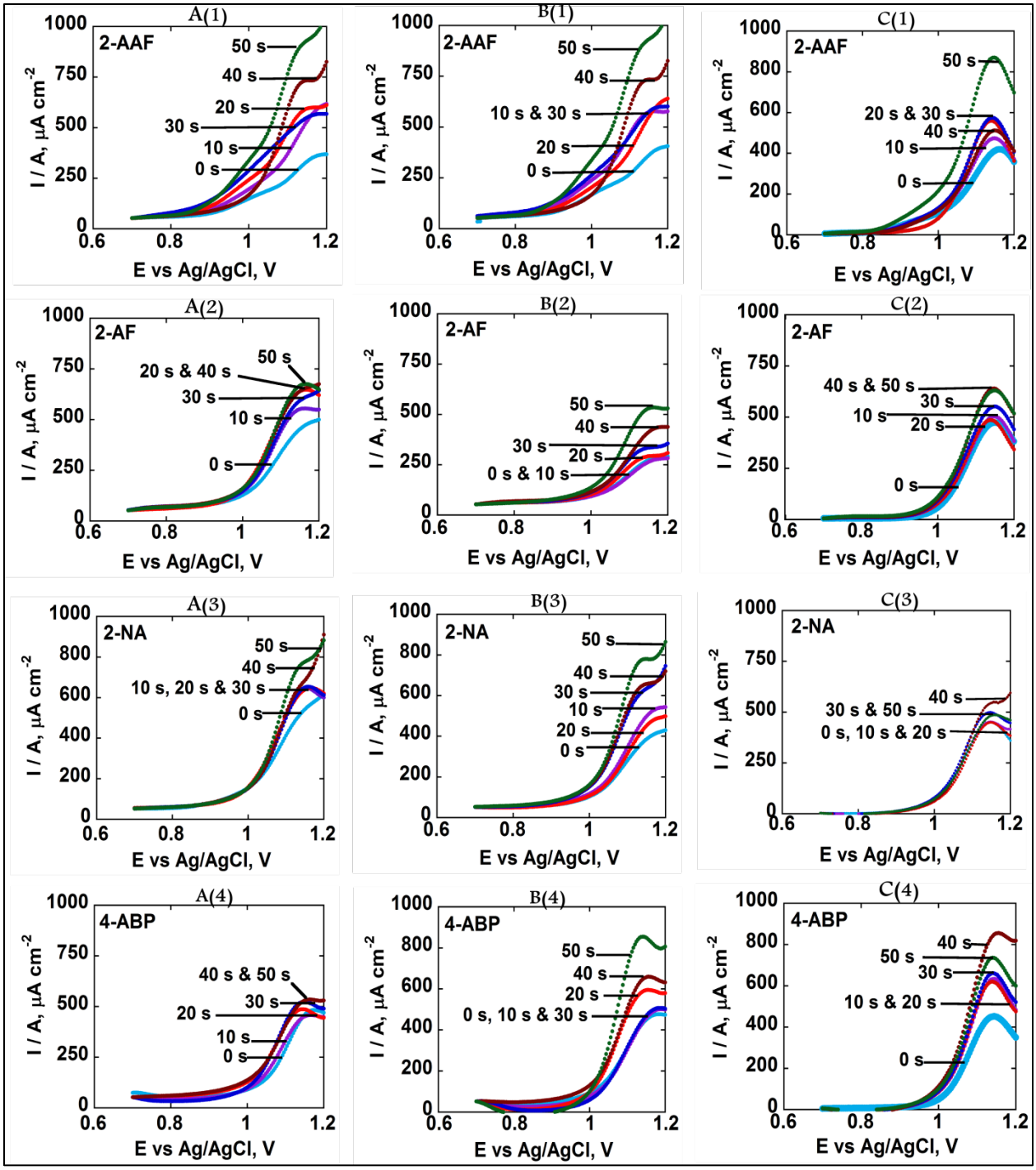

Fig. S7. Background subtracted average difference SWV peak current densities measured sequentially at different enzyme reaction times in anaerobic pH=5.8 10 mM acetate buffer for multiplexed array sensors featuring optimized films of (A) HLM, (B)HLM+NAT, (C) Hs9, (SWV ampl. 25 mV; freq. 15 Hz; step 4 mV) for the exposure of (1) 0.25 mM 2-AAF, (2) 0.2 mM 2-AF, 2) 0.2 mM 2-NA, (4) 0.05 mM 4-ABP/0.01 mM 4-ABP.

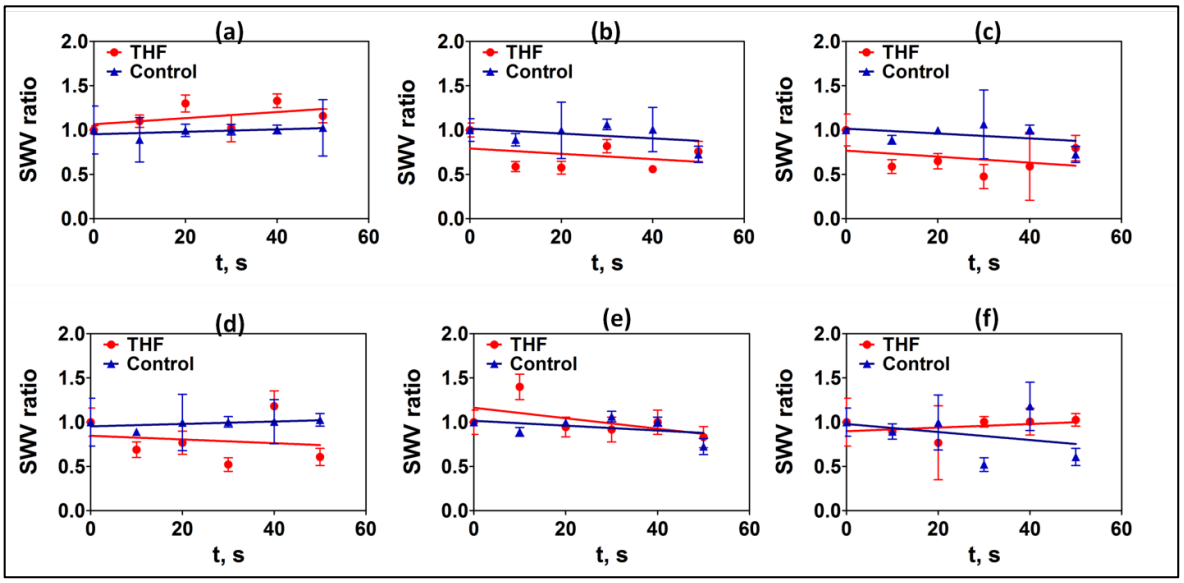

Fig. S8. Influence of substrate incubation time on SWV peak current ratio ($I_{p,f}/I_{p,i}$) for multiplexed microfluidic genotoxicity sensor arrays of (a) HLM, (b) HLM+NAT, (c) HLM+NAT, (d) 1B1, (e) 1B1+EH, (f) Hs9 for the exposure of 0.2 mM THF at pH 7.4 phosphate buffer. Controls are incubations without THF or exposure to the THF without electrolysis, which gave equivalent results. Error bars represent standard errors for n=4.

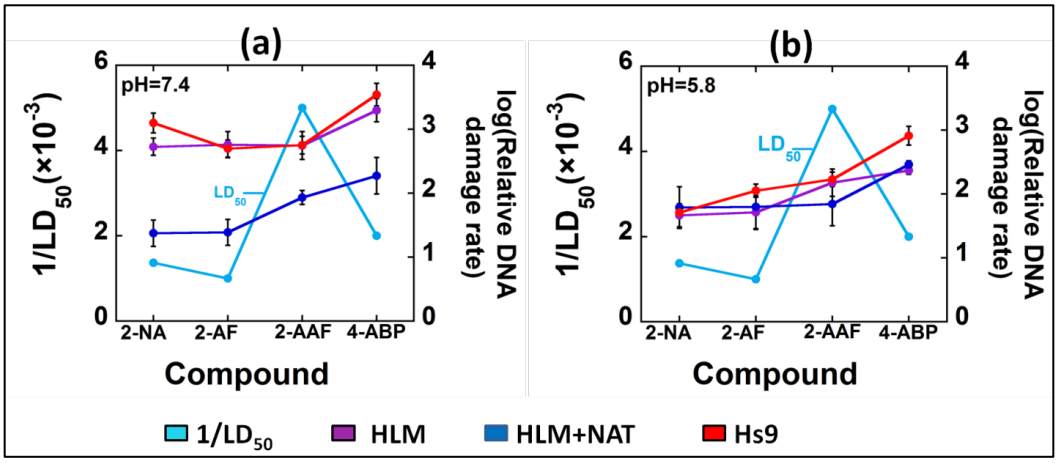

Fig. S9. Plots illustrating correlations of the reciprocal of rodent LD_{50} values with log of relative DNA damage rates ({μg protein^{-1}} s^{-1} mM^{-1}) measured by sensor arrays containing HLM, HLM+NAT and Hs9 as enzyme sources, for 4 arylamines (a) at pH 7.4 and (b) at pH 5.8.

References

1 R. Sato and T. Omura, *J. Biol. Chem.,*1964, **239**, 2370-2378.
2 Y. Lvov, in *Handbook of Surfaces and Interfaces of Materials*, ed. R. W. Nalwa, Academic Press, San Diego, CA, 2001, Vol. 3, pp 170-189.
3 P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk, *Anal. Biochem.,* 1985, **150**, 76-85.