Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information

An automated microfluidic gene-editing platform for deciphering cancer genes

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Device fabrication and assembly

Digital microfluidic devices were fabricated following methods described previously (Fig. S4).[1, 2] Briefly, designs were drawn using AutoCAD 2015 (Autodesk, San Rafael, CA) and photomasks were then printed in high-resolution (20,000 dpi) by CAD/Art Services Inc (Bandon, OR). The bottom-plates bearing patterned electrodes were formed by standard photolithography techniques, in the Concordia Silicon Microfabrication Lab (ConSIM). Chromium substrates coated with photoresist were UV-exposed through the photomask (7 s, 42.4 mW/cm^2) to imprint the transparency mask designs. Substrates were then developed in MF-321 positive photoresist developer (2 min, shaking), rinsed with DI water, dried under a stream of nitrogen and baked for 1 min at 115 °C. The exposed chromium was then etched using CR-4 chromium etchant (3 min) and substrates were then rinsed with DI water and dried under a stream of nitrogen. Finally, devices were immersed in AZ300T photoresist stripper (3 min) to remove any remaining photoresist before being rinsed and dried under a stream of nitrogen. Once the patterning step was completed, the substrates were immersed in a silane solution consisting of deionized water, isopropanol and 3-(Trimethoxysilyl)propyl-methacrylate (50:50:1) for dielectric priming during 15 min. Substrates were rinsed with isopropanol, DI water and then dried under a stream of nitrogen. Prior to the addition of the polymer coatings to complete the process, thermal tape was added on top of the contact pads to facilitate later removal of the polymer coatings from the contact pads and allow electrical contact for droplet actuation. Parylene-C was used as a dielectric which was deposited by chemical vapor deposition in a SCS Labcoter 2 PDS 2010 (Specialty Coating Systems, Indianapolis, IN) achieving a homogenous final thickness of 7 μm. FluoroPel PFC1601V was used as a hydrophobic coating and was spin-coated in a Laurell spin-coater at 1500 rpm for 30 s followed by post-baking on a hot-plate (180 °C, 10 min).

The DMF top-plates consist of a continuous ground electrode formed from an indium tin oxide (ITO) coated glass substrate. For typical ground plates, ITOs were spin-coated with the FluoroPel PFC1601V using the same program as described in the bottom-plate fabrication procedure. ITOs bearing an array of hydrophilic spots (i.e., circular regions of exposed ITO) for on-chip tissue culture were microfabricated using a fluorocarbon-liftoff procedure (following procedures described previously.[3, 4] ITOs were cleaned by immersion in an RCA solution comprising of DI water, 28% aqueous ammonium hydroxide, 30% hydrogen peroxide (5:1:1 v/v/v) for 30 min at 80 °C on a hotplate. After rinsing, drying and dehydrating (2 min at 95°C), the substrates were spin-coated with Shipley S1811 photoresist (10 s, 500 rpm, ACL=100 rpm and 60 s, 3000 rpm, ACL=500 rpm) and baked at 95 °C for 2 min. Slides were cut to the desired size (i.e.: 50 x 75 mm) using a Cuter's Mate (Creator's Stained Glass, Victoria, BC) and vented under a stream of nitrogen. Substrates were exposed through the photomask with an array of six 1.75 mm diameter circular features (10 s, 42.4 mW/cm^2) and developed in MF-321 (3 min). After rinsing, air-drying and dehydrating (1 min, 95°C), top-plates were then flood exposed (10 sec, 42.4 mW/cm^2), spin-coated with 1% Teflon in FC-40 (10 s, 500 rpm, ACL = 100 rpm and 60 s, 3000 rpm, ACL = 500), and post-baked on a hotplate (165 °C, 10 min). After allowing to cool on aluminum foil for 2 min, substrates were immersed in acetone with gentle agitation for 10-15 s until the Teflon-AF over the patterned sites was lifted off. After being rinsed with DI water and dried under a stream of nitrogen, droplets of AZ300T stripper was gently placed over the spots and substrates were placed aside for 1 min followed by rinsing with DI water and air-drying. Post-baking followed to reflow the Teflon-AF at 165°C, 210°C and 300°C for 5 min at each temperature.

Complete devices were assembled with the continuous ground ITO top-plate and the chromium electrode-bearing bottom plate, being joined by stacking two layers of double sided tape to a gap height of approximately 140 μm. Alignment of the ITO top plate above the bottom plate was performed with care such that the edge of the top plate was adjacent to the outer-edges of the reservoir electrodes of the bottom-plate pattern (see Fig. 1a). Moreover, each 25 mm x 75 mm top plate was roughly aligned to the electrodes over which the virtual microwells were required.

Automation setup and device operation

The automation system (Fig. S5) consists of a MATLAB (Natlick, MA) program that is used to control an Arduino Uno microcontroller (Adafruit, New York, USA). Driving input potentials of 130-270 V_{RMS} were generated by amplification of a sine wave output from a function generator (Agilent Technologies, Santa Clara, CA) operating at 10 kHz by a PZD-700A amplifier, (Trek Inc., Lockport, NY) and delivered to the PCB control board. The Arduino controls the state of high-voltage relays (AQW216 Panasonic, Digikey, Winnipeg, MB) that are soldered onto the PCB control board. The logic state of an individual solid-state switch is controlled through an I^2C communication protocol by an I/O expander (Maxim 7300, Digikey, Winnipeg, MB). This control board is mated to a pogo pin interface (104 pins), where each switch delivers a high-voltage potential (or ground) signal to a contact pad on the DMF device. See our GitHub registry (https://github.com/shihmicrolab/Automation) to assemble the hardware and to install the open-source software program to execute the automation system.

To start gene-editing experiments, reagent loading was achieved by pipetting a droplet of liquid onto the outer-edge of a reservoir electrode and adjacent to the gap between the bottom and top plates and actuating the reservoir electrode. Once inside the reservoirs, the droplets were then

actively dispensed, moved, mixed or merged by sequential actuation of neighboring electrodes on the bottom plates in air as the filler medium. Active dispensing was achieved over three electrodes and results in a droplet with a diameter of the same size as the electrodes (i.e. a unit droplet). To initiate passive dispensing, it was achieved by moving an actively dispensed droplet over the vacant lift-off spot. At times, contents on this spot may be displaced with the contents of a new source droplet. Generally, all droplets containing proteins were supplemented with 0.05% Pluronics F-68. Waste and unused fluids were removed by delivering them to reservoirs and removed using KimWipes (Kimberly-Clark, Irving, TX).

Table S1 – Cells and Plasmids used in this study

Cells	**Genotype**	**Source**
E. coli DH5α	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	V. Martin
Cell Line	**Transgene Integration**	**Source**
NCI-H1299 (*Human lung squamous cell carcinoma dual-labeled stable)*	KanR	Genecopoeia SL001
Plasmids	**Relevant characteristics**	**Addgene #**
mCherry2-N1	KanR	54517
All_in_one_CRISPR/Cas9_LacZ	AmpR	74293
pSpCas9(BB)-2A-Puro (PX459) v2.0	AmpR, PuroR	62988

Custom pCRISPR Plasmids	**Custom Sequence**	**PAM**	**Source**
pCRISPR_eGFP_191	– / ACTGCACGCCGTAGGTCAGGG	TGG	This study
pCRISPR_eGFP_314	+ / GCAACTACAAGACCCGCGCCG	AGG	This study
pCRISPR_eGFP_369	+ / TCGATGCCCTTCAGCTCGATG	CGG	This study
pCRISPR_eGFP_497	+ / TCAAGATCCGCCACAACATCG	AGG	This study
pCRISPR_eGFP_683	– / CCATGCCGAGAGTGATCCCGG	CGG	This study
pCRISPR_RAF1_94	+ / GCCGCCCGAGAGTCTTAATCG	CGG	This study
PX459_eGFP_12-31	+ / GGGCGAGGAGCTGTTCACCG	GGG	Genscript

Table S2 – Primer Sequences

Gene	**Orientation**	**Sequence**
g-block_universal	Forward	ATATATCGTCTCGAACTTGAAAGTATTTCGATTTCTTGGGT
g-block_universal	Reverse	ATAATTCGTCTCTAGCGCAAAACGCCTAACCCTAAGCAGATTCTTCATGCAATTGTGTCTAGAAAAAAGCACCGACTCGGTG
SP6 sequencing primers	Forward	ATTTAGGTGACACTATAG

sgRNA 438 bp

5' – ATTCCCCAGTGGAAAGACGCGCAGGCAAAACGCACCACGTGACGGAGCGTGA
CCGCGCGCCGAGCGCGCGCCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTC
CTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGAC
TGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGG
TAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTG
AAAGTATTTCGATTTCTTGGGTTTATATATCTTGTGGAAAGGACGAggatcNNNNNNNNN
NNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTA
TCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT **– 3'**

Fig. S1. The sgRNA sequence represents the template designed for all sgRNAs. It consists of the U6 Promoter, the variable seed sequence, the dCas9 handle and the S. pyogenes terminator. The seed sequences varied according to the target region (see Table S1). All eight constructs were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

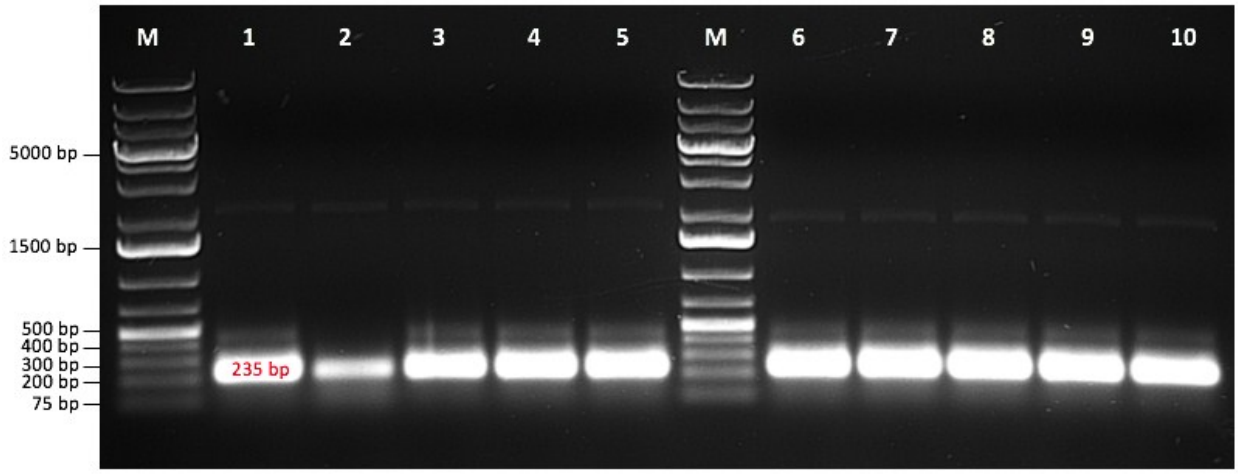


Fig. S2 - Gel electrophoresis image of the PCR products of the synthesized CRISPR guides, yielding g-blocks. PCR products were loaded into a 0.8% agarose gel in TAE buffer and resolved at 130 V for 30 min. These represent the g-blocks flanked with BsmBI cut sites, ready for insertion into a pCRISPR backbone. (1) KRAS_5608; (2) KRAS_41162; (3) RAF1_94; (4) RAF1_253; (5) RAF1_64486; (6) EGFP_191; (7) EGFP_314; (8) EGFP_369; (9) EGFP_497; (10) EGFP_683.

Fig. S3 - Blue/white screening. A schematic showing the procedure of inserting a CRISPR guide into a Cas9 vector backbone. An all-in-one pCRISPR template tailored to blue-white screening was used. The LacZα open reading frame, necessary to complement Δ(lacZ)M15 for functional beta-galactosidase expression, was inserted between two BsmBI flanking sites. One-pot assembly reactions containing the all-in-one pCRISPR template, the restriction enzymes, the g-block and the T4 DNA ligase were placed in a thermal cycler and the product was transformed into E. coli. Cells were plated on LB Agar with S-Gal, a colorless substrate that gets hydrolyzed by beta-galactosidase and results in blue bacterial colonies. Cells that were transformed with recombinant vectors of interest would be white, and those transformed with non-recombinant vectors would be blue.

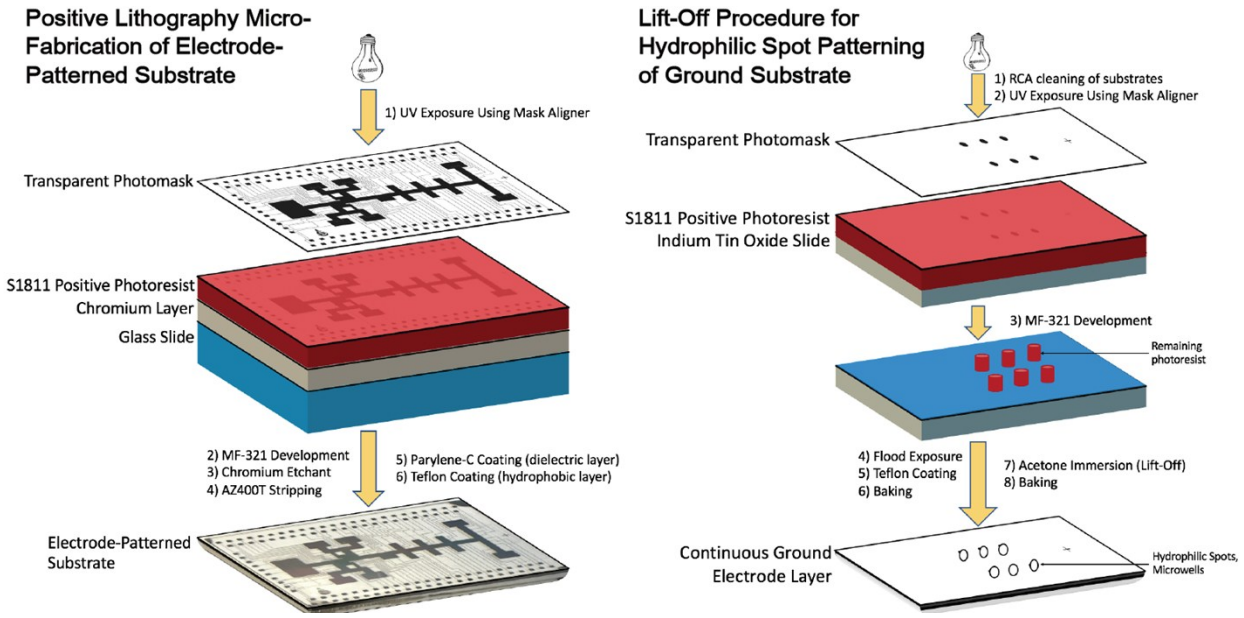


Fig. S4 - Schematic of DMF device and top-plate fabrication. Bottom-plate fabrication followed a photolithography procedure (left) and top-plate fabrication followed a standard lift-off procedure (right).

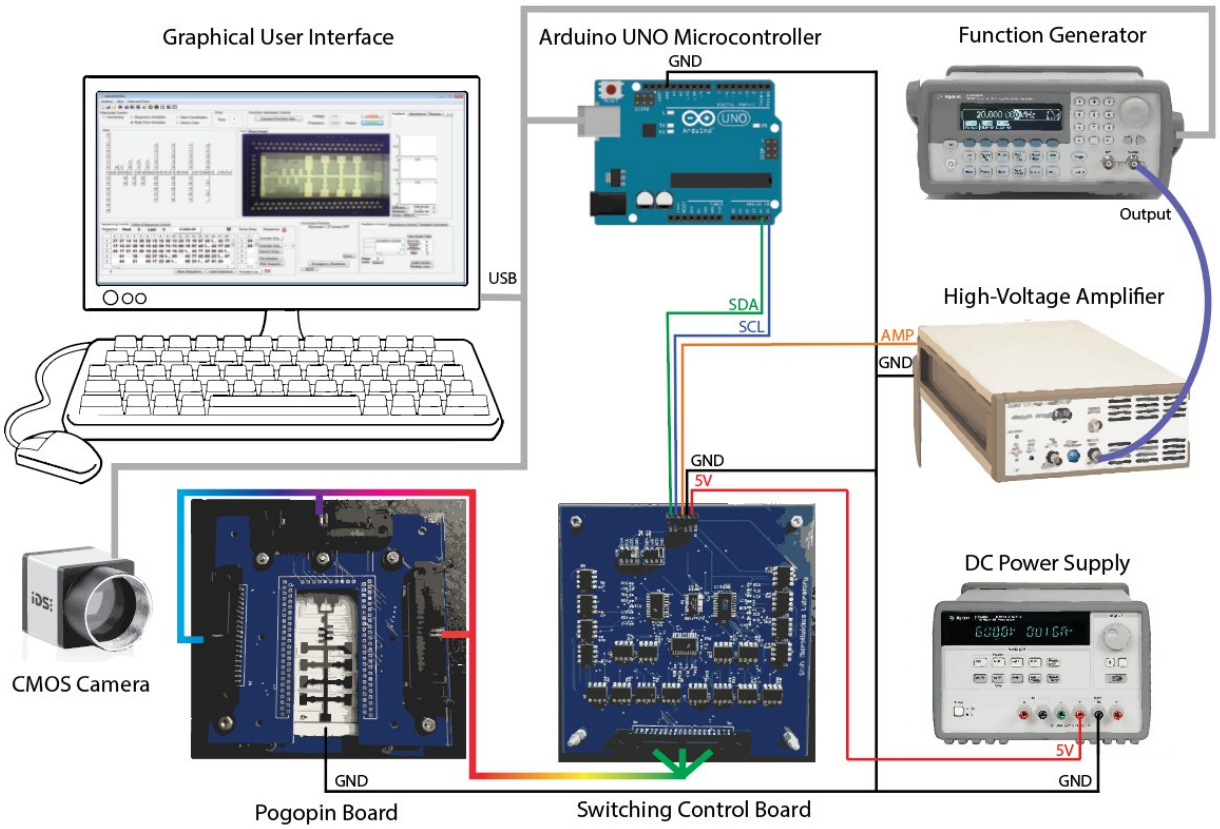


Fig. S5 - Microfluidic automation system for gene-editing. The automation system consists of a custom MATLAB program interfaced to an Arduino Uno microcontroller. The Arduino controls the state of high-voltage relays on a switching control board. Sine waves are generated from a function generator operating at 10 kHz and amplified using a high-voltage amplifier, producing driving input potentials of 130-270 V_{RMS} to the control board. The control of the state of an individual switch is done through an I^2C communication protocol using an I/O expander. The control board is mated to a pogo pin board, where each switch is wired to an individual pogo-pin, in contact with a contact pad. The device is imaged live through a CMOS camera.

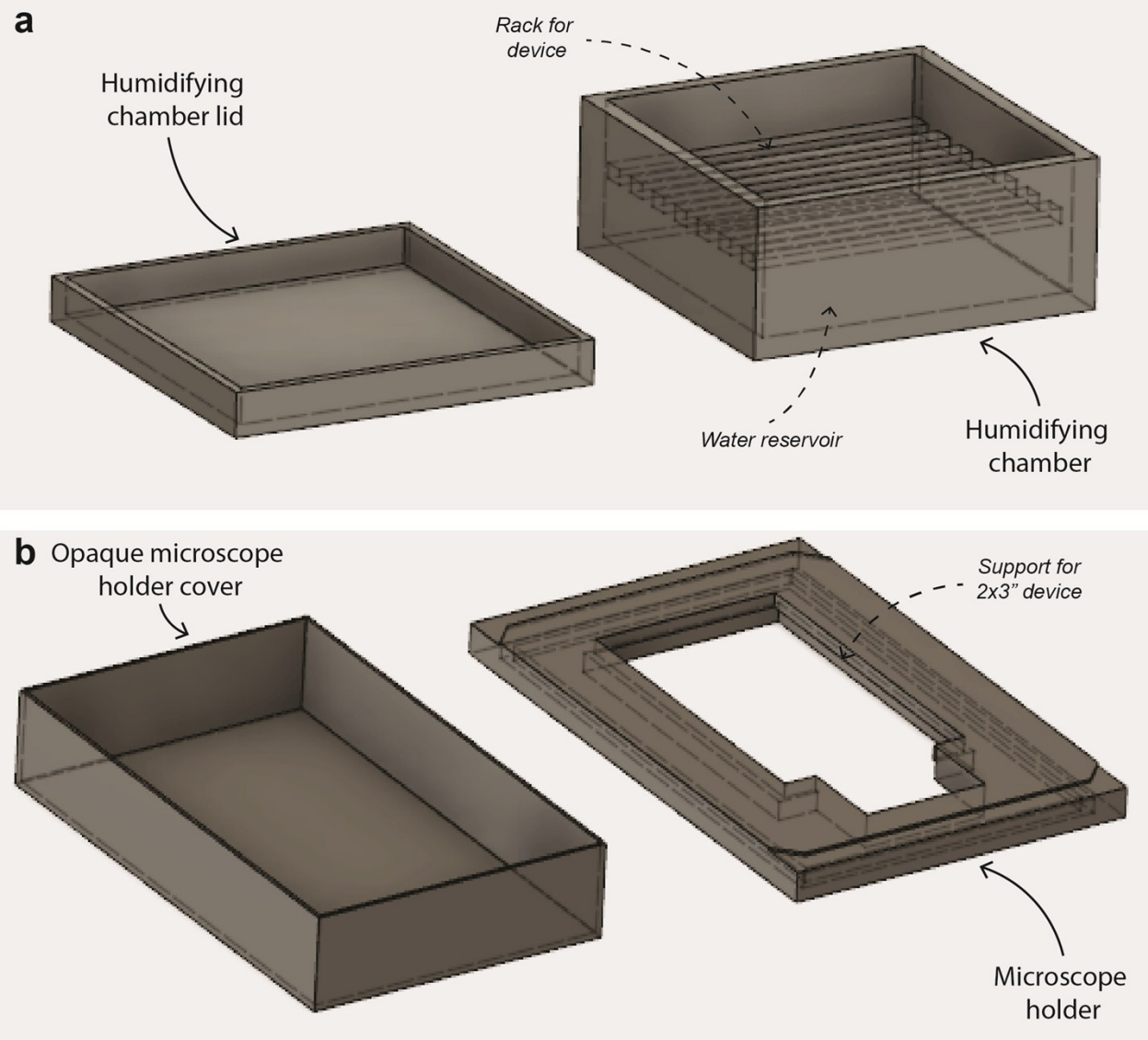


Fig. S6 - 3D-printed humidified chamber and microscope holder for imaging. (A) Cell humidified chamber with cover to prevent evaporation of droplets. The design consists of a rack above a water reservoir, on which the devices are placed and of a lid to prevent evaporation and enable saturation in humidity. (B) Microscope holder tailored to digital microfluidic devices, with opaque cover for fluorescence microscopy.

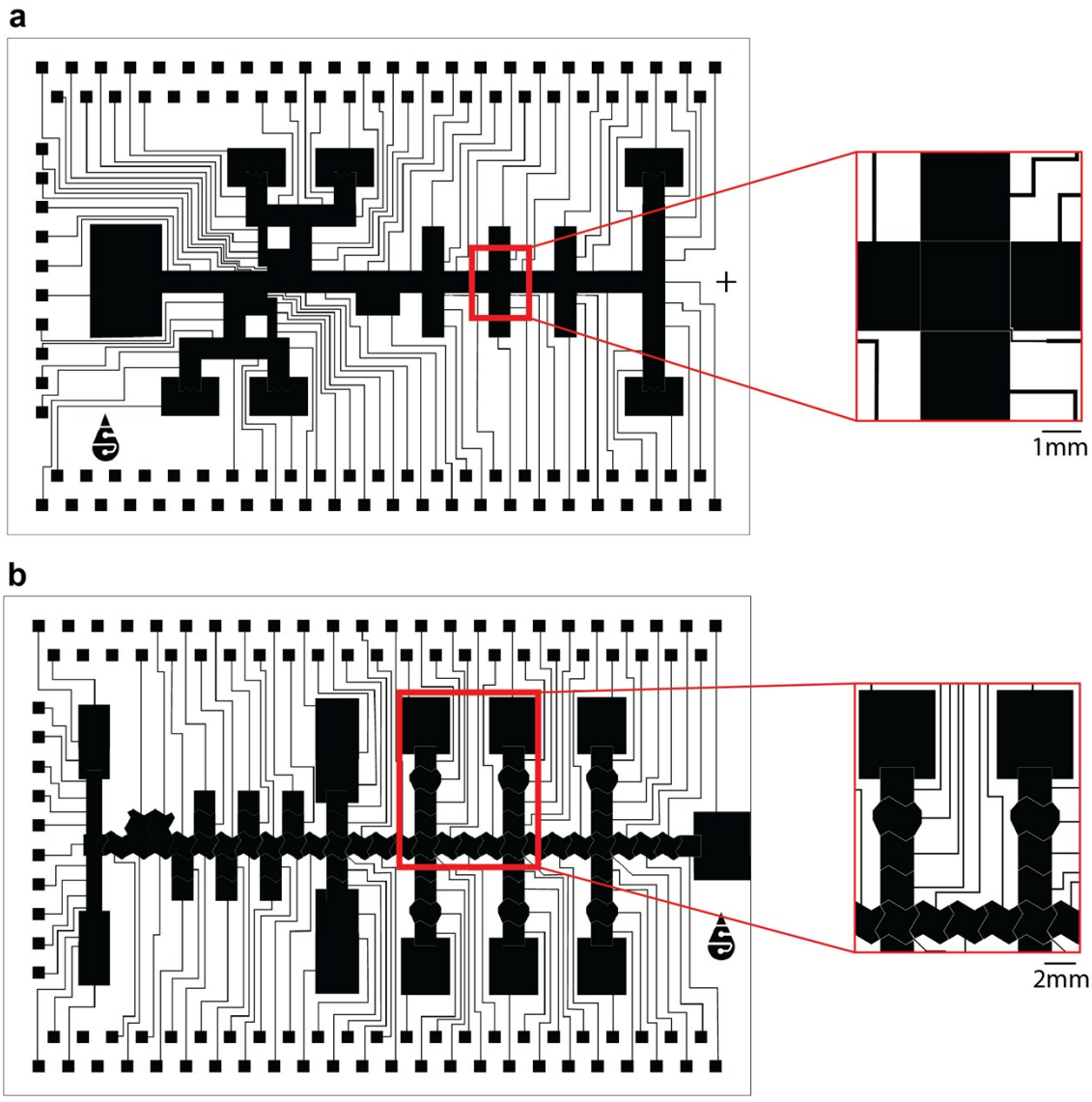


Fig. S7 - Optimization of chip configuration and electrode design. (a) The first design shows a configuration with square electrodes. (b) The current design is modified to have interdigitated electrodes to facilitate droplet movement.

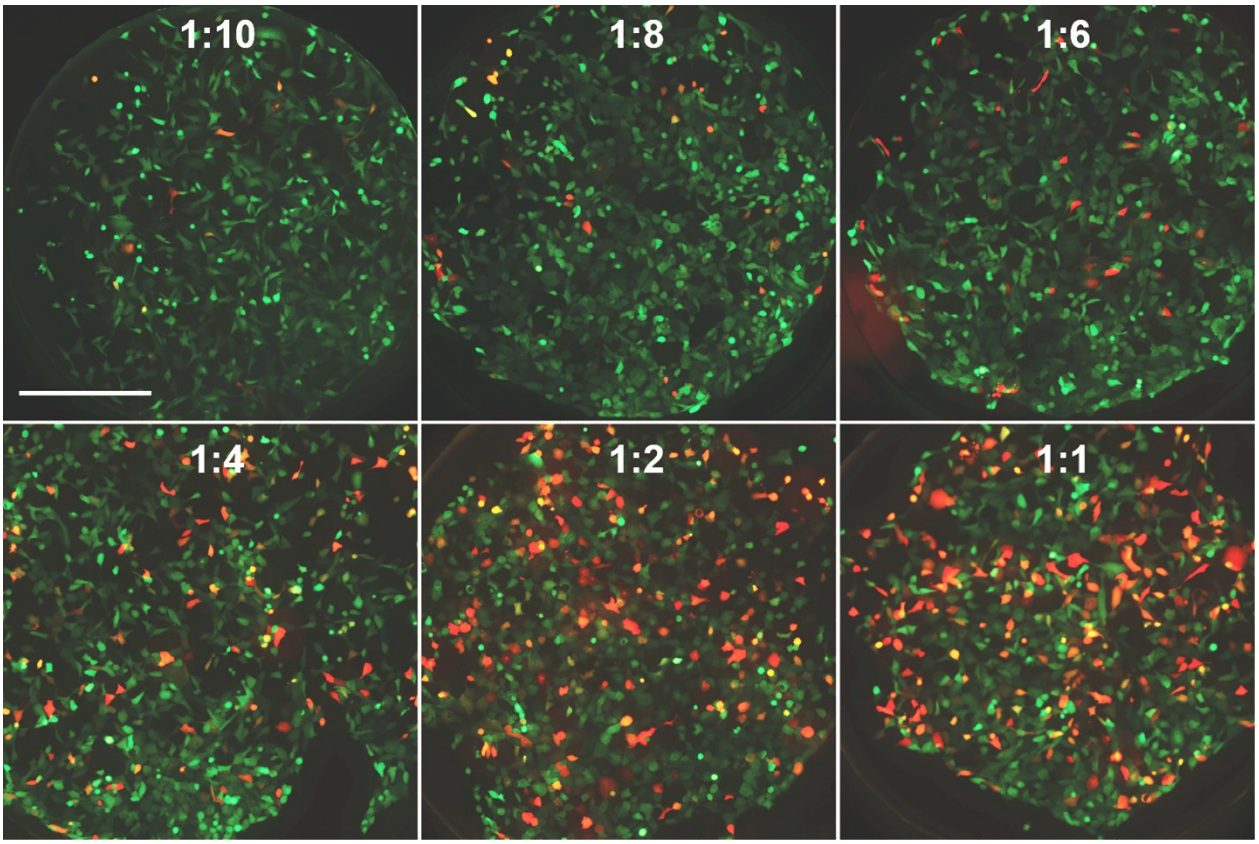


Fig. S8 - Optimization of on-chip transfection using various dilutions of lipid complexes in liquid media. Overlapped eGFP and mCherry images show empirical transfection efficiencies for a range of different ratios (1:10, 1:8, 1:6, 1:4, 1:2, 1:1). The 1:1 ratio shows highest transfection efficiency. Scale bar = 0.5 mm.

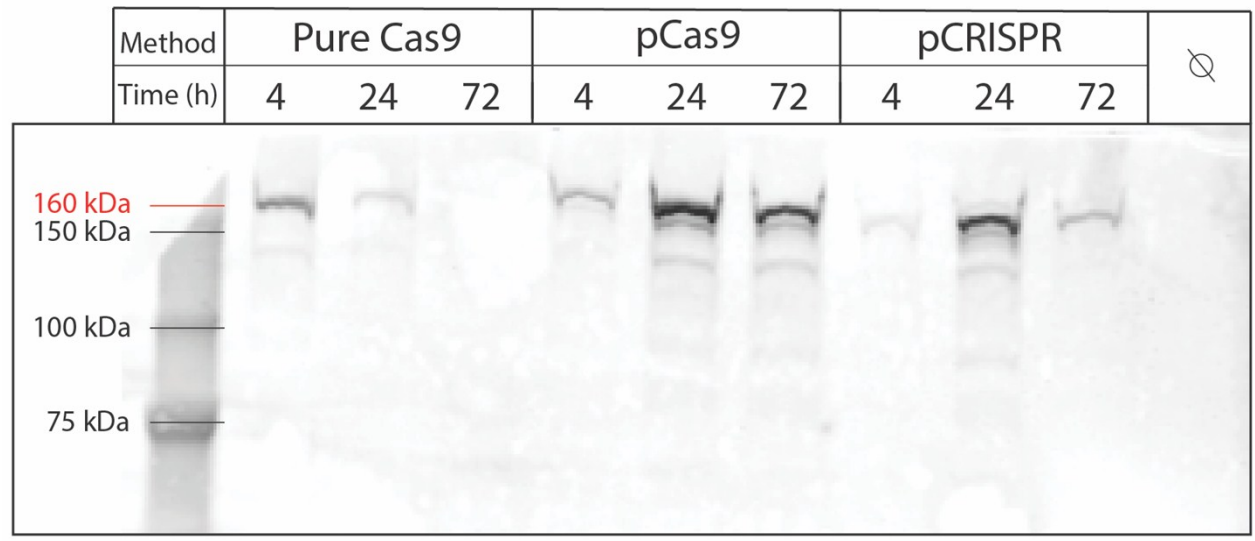


Fig. S9 - Western Blot showing Cas9 protein levels comparing different starting material of Cas9 into NCI-H1299 cells. Lipid-mediated transfection was done using three different starting materials (DNA and protein), and lysates were collected at three different time-points (4, 24, and 72 h). Lane (1) shows pure Cas9 protein to assess transfection of RNP complexes. Lane (2) shows Cas9 expressing plasmid, pCas9, to assess co-transfection of pCas9 with an sgRNA plasmid. Lane (3) shows transfection of pCRISPR all-in-one plasmid (Cas9 and sgRNA). A negative control was transfected with the mCherry2-N1 plasmid and the lysate was collected after 24 h. The expected protein size of Cas9 is 160 kDa which is highlighted in red.

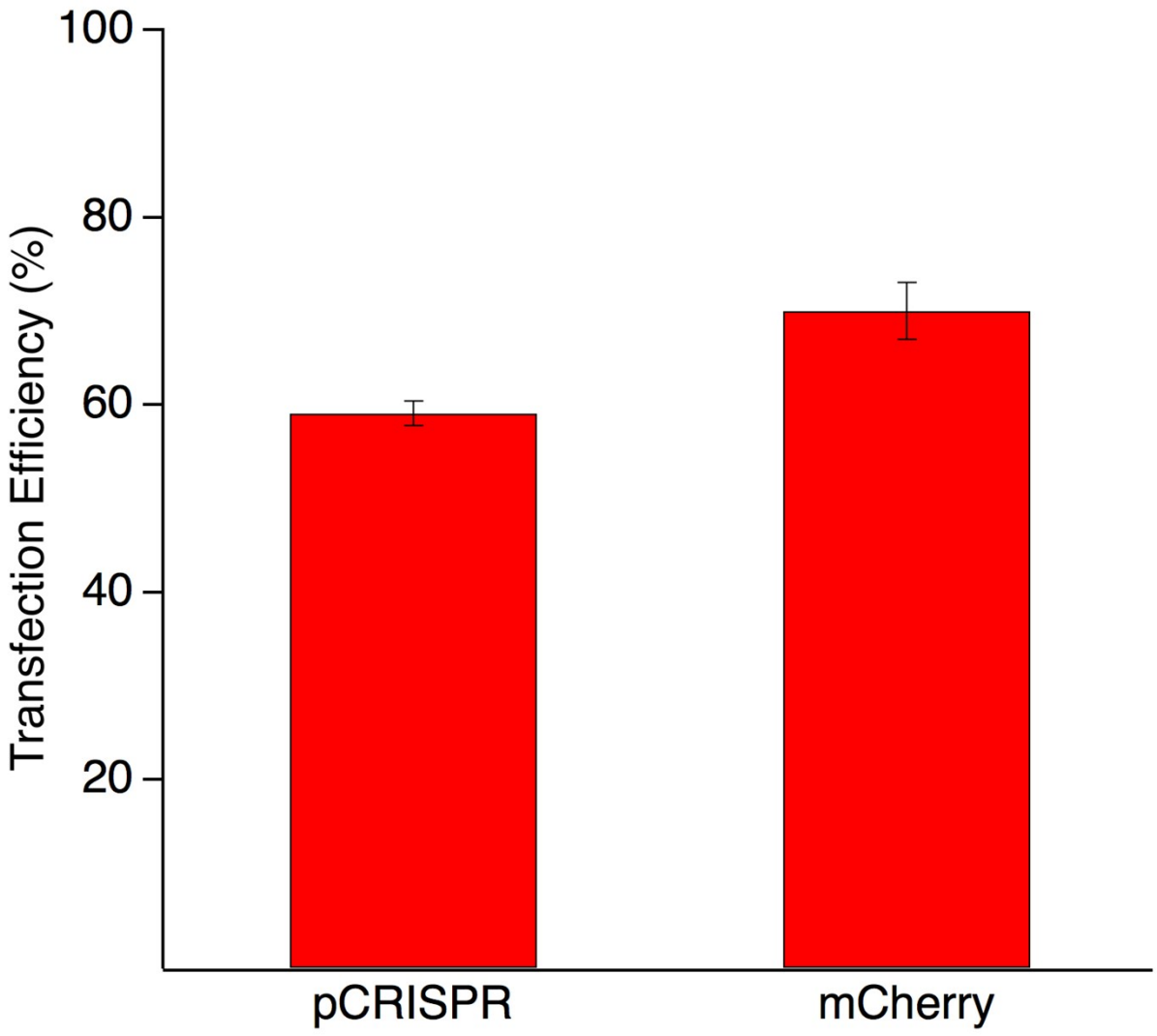


Fig. S10 - Plot of the transfection efficiency for both the All_in_one_CRISPR/Cas9_LacZ (pCRISPR) and mCherry2-N1. pCRISPR has a reporter mCherry gene under an SV40 promoter, and a CMV promoter was used for the mCherry plasmid. For transfection, a 1:10 ratio of lipid complexes to media was used. Images of the transfected H1299 cells were taken after 48 h and processed using the standardized transfection pipeline.

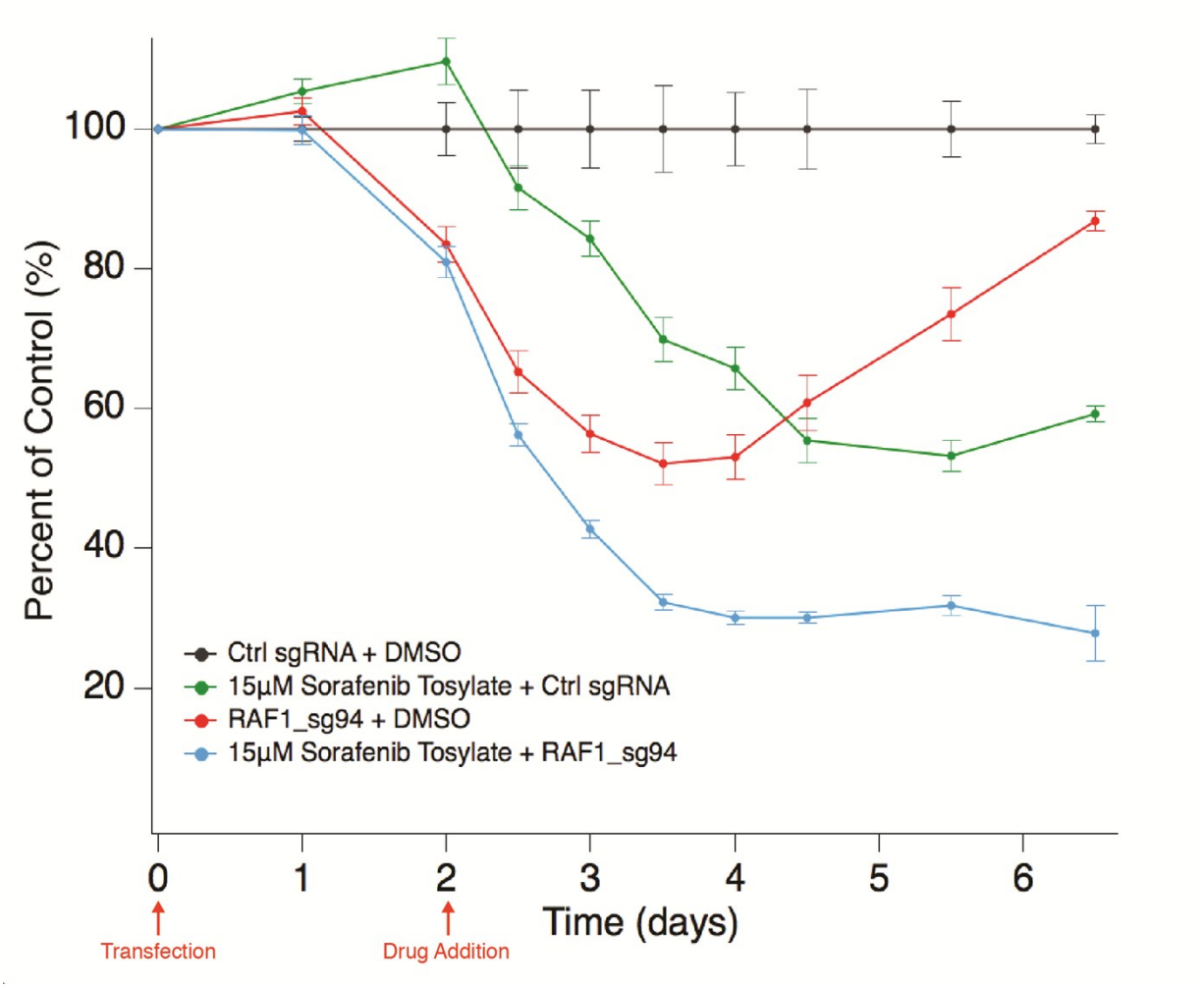


Fig. S11 - Plot showing progression of cell viability over time. Four conditions were tested by acquiring fluorescent measurements over 7 days to assess proliferation. Cells were transfected on day 0 with an sgRNA targeting RAF1 or a scramble sgRNA. After 48 h post-transfection, a drug Sorafenib Tosylate or DMSO and was added to the guides. All readings were taken in triplicate and error bars represent ± 1 s.d.

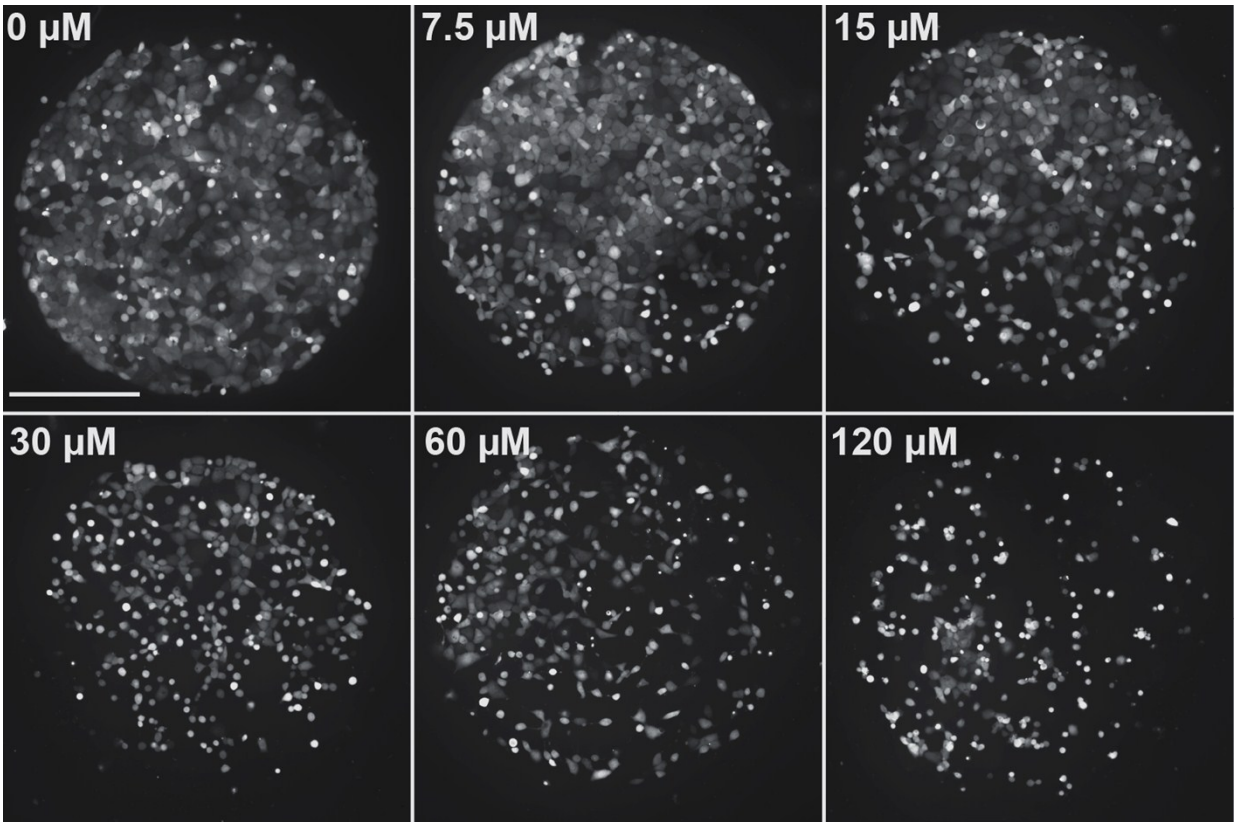
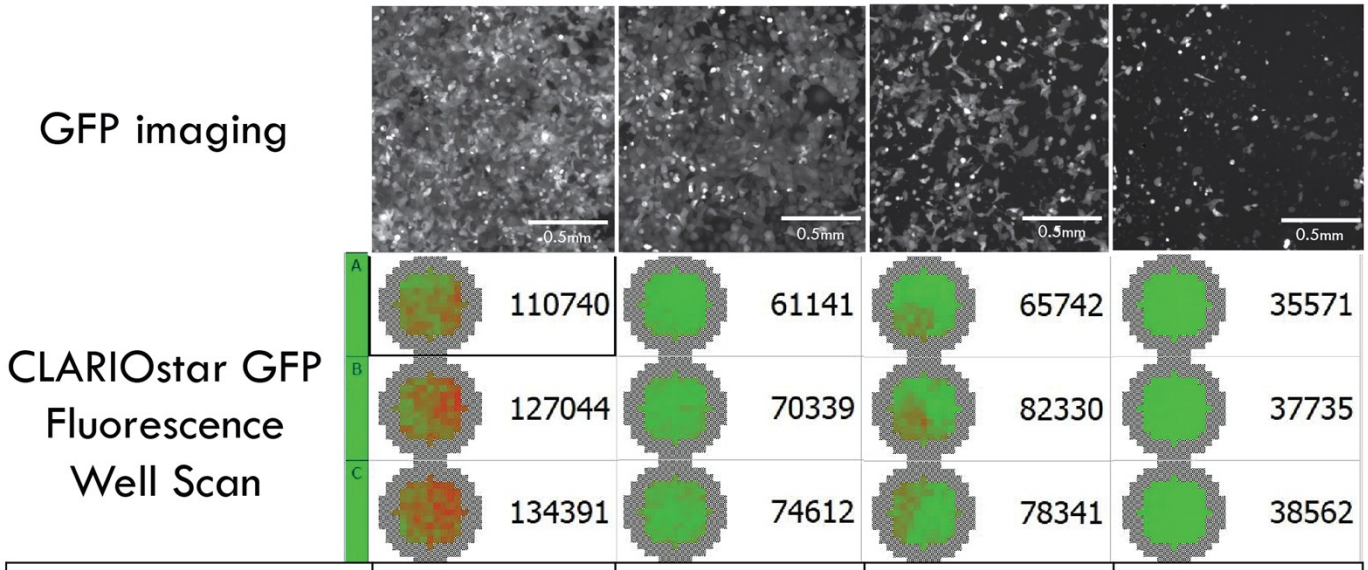


Fig. S12 - Microscopy images of H1299 cells on-chip. Each image shows a condition that is treated with the enzymatic inhibitor Sorafenib Tosylate**.** The images are taken on day 5. Scale bar = 0.5 mm.



sgRNA Scramble	+	+	-	-
sgRNA vs. RAF1	-	-	+	+
Sorafenib Tosylate 15μM	-	+	-	+

Fig. S13 - Raw data showing the absolute fluorescence and the morphology of the H1299 cells. Four conditions were tested and microscopy fluorescent images were captured on day 5 using GFP filter set.

References:

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2. M. C. Husser, P. Q. N. Vo, H. Sinha, F. Ahmadi and S. C. C. Shih, *ACS Synth Biol*, 2018, **7**, 933-944
3. A. H. C. Ng, M. D. Chamberlain, H. Situ, V. Lee and A. R. Wheeler, *Nat Commun*, 2015, **6**. 7513
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