Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59655R2
Full Title:	A Multilayer Microfluidic Platform for the Conduction of Prolonged Cell-Free Gene Expression
Keywords:	Microfluidics, in vitro transcription and translation, synthetic biology, prolonged reactions, micro-reactors, protein expression
Corresponding Author:	Tom F. A. de Greef Technische Universiteit Eindhoven Institute for Complex Molecular Systems Eindhoven, NETHERLANDS
Corresponding Author's Institution:	Technische Universiteit Eindhoven Institute for Complex Molecular Systems
Corresponding Author E-Mail:	t.f.a.d.greef@tue.nl
Order of Authors:	Ardjan J. van der Linden
	Maaruthy Yelleswarapu
	Pascal A. Pieters
	Zoe Swank
	Wilhelm T. S. Huck
	Sebastian J. Maerkl
	Tom F. A. de Greef
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Eindhoven, Noord Brabant, The Netherlands

1 **TITLE:**
2 A Multilayer Microfluidic Platform for the Conduction of Prolonged Cell-Free Gene Expression
3

4 **AUTHORS & AFFILIATIONS:**
5 Ardjan J van der Linden[1], Maaruthy Yelleswarapu[2], Pascal A Pieters[1], Zoe Swank[3], Wilhelm T S
6 Huck[2], Sebastian J Maerkl[3*], Tom F A de Greef[1,2*]
7

8 [1]Institute for Complex Molecular Systems, Department of Biomedical Engineering,
9 Computational Biology Group, Eindhoven University of Technology, Eindhoven, The Netherlands
10 [2]Institute for Molecules and Materials, Radboud University, Nijmegen, The Netherlands
11 [3]Institute of Bioengineering, School of Engineering École Polytechnique Fédérale de Lausanne
12 (EPFL), Lausanne, Switzerland
13

14 **CORRESPONDING AUTHORS:**
15 Tom F A de Greef
16 T.F.A.d.Greef@tue.nl
17 Sebastian J Maerkl
18 sebastian.maerkl@epfl.ch
19

20 **Email Addresses of Co-authors**:
21 Ardjan van der Linden   (a.j.v.d.linden@tue.nl)
22 Maaruthy Yelleswarapu   (m.Yelleswarapu@science.ru.nl)
23 Pascal Pieters   (p.a.pieters@tue.nl)
24 Zoe Swank   (zoe.Swank@epfl.ch)
25 Wilhelm Huck   (w.huck@science.ru.nl)
26

27 **KEY WORDS:**
28 Microfluidics, in vitro transcription and translation, synthetic biology, prolonged reactions, micro-
29 reactors, protein expression
30

31 **SUMMARY:**
32 The fabrication process of a PDMS-based, multilayer, microfluidic device that allows in vitro
33 transcription and translation (IVTT) reactions to be performed over prolonged periods is
34 described. Furthermore, a comprehensive overview of the hardware and software required to
35 automate and maintain these reactions for prolonged durations is provided.
36

37 **ABSTRACT:**
38 The limitations of cell-based synthetic biology are becoming increasingly apparent as researchers
39 aim to develop larger and more complex synthetic genetic regulatory circuits. The analysis of
40 synthetic genetic regulatory networks in vivo is time consuming and suffers from a lack of
41 environmental control, with exogenous synthetic components interacting with host processes
42 resulting in undesired behavior. To overcome these issues, cell-free characterization of novel
43 circuitry is becoming more prevalent. In vitro transcription and translation (IVTT) mixtures allow
44 the regulation of the experimental environment and can be optimized for each unique system.

45 The protocols presented here detail the fabrication of a multilayer microfluidic device that can
46 be utilized to sustain IVTT reactions for prolonged durations. In contrast to batch reactions,
47 where resources are depleted over time and (by-) products accumulate, the use of microfluidic
48 devices allows the replenishment of resources as well as the removal of reaction products. In this
49 manner, the cellular environment is emulated by maintaining an out-of-equilibrium environment
50 in which the dynamic behavior of gene circuits can be investigated over extended periods of time.
51 To fully exploit the multilayer microfluidic device, hardware and software have been integrated
52 to automate the IVTT reactions. By combining IVTT reactions with the microfluidic platform
53 presented here, it becomes possible to comprehensively analyze complex network behaviors,
54 furthering our understanding of the mechanisms that regulate cellular processes.
55
56 **INTRODUCTION**
57 Cells are able to sense and respond to their environment using complex dynamic regulatory
58 networks[1,2]. The field of synthetic biology utilizes our knowledge of the naturally occurring
59 components comprising these networks to engineer biological systems that can expand the
60 functionality of cells[3,4]. Conversely, it is also possible to further our understanding of the natural
61 networks governing life by designing simplified, synthetic analogues of existing circuits or by
62 forward-engineering biological systems which exhibit naturally occurring behaviors. The de novo
63 engineering of such biological systems is performed in a bottom-up fashion where novel genetic
64 circuits or signalling pathways are engineered in a rational manner, using well-defined parts[5,6].
65 Combining the rational design of networks with the design of biologically relevant systems
66 enables the in-depth characterization and study of biological regulatory systems with various
67 levels of abstraction[7].
68
69 The pioneering works of Elowitz and Leibler[8] and Gardner et al.[9] were the first to demonstrate
70 the successful introduction of synthetic genetic networks into cellular hosts. In the following
71 decade, numerous researchers have continued to build on these initial successes despite the
72 emergence of several limitations regarding the introduction of synthetic circuits into cells[7,10,11,12].
73 Ideally, the introduction of synthetic circuits into cellular hosts should occur in a modular fashion.
74 Unfortunately, the complexity of the cellular environment makes this particularly challenging,
75 with the function of many parts and networks being highly context dependent[12,13,14]. As a result,
76 networks often experience undesired interactions with native host componentry which can
77 affect the function of the synthetic circuit. Similarly, components of the exogenous network can
78 inhibit host processes, compete for shared resources within the host, and influence growth
79 kinetics[15–17]. Consequently, in order to rationally design and predict the behavior of synthetic
80 networks in an in vivo environment, a comprehensive model of all host and circuit-specific
81 dynamics is required[18].
82
83 A viable alternative to the use of cellular hosts for the characterization of synthetic networks is
84 the application of in vitro transcription and translation (IVTT) technologies. Acting as a testbed
85 for synthetic networks, reactions are performed in solutions comprising all the components
86 required to enable gene expression[19–21]. In this manner, a biologically relevant, albeit artificial,
87 environment is created within which synthetic networks can be tested[22–28]. A major advantage
88 of using IVTT solutions is the ability to perform reactions under user-specified conditions, with

89   researchers able to tune the precise composition of each reaction[2]. Furthermore, the cell-free
90   approach enables high-throughput testing of synthetic networks, since it removes the need to
91   perform time-consuming cellular cloning steps. As a result, the duration of successive design –
92   build – test cycles is significantly reduced[29–32]. The design cycle can be further accelerated by
93   utilizing cell-free cloning techniques such as the Gibson assembly to rapidly engineer novel
94   networks, and by constructing networks from linear DNA templates which – unlike the plasmids
95   required for in vivo testing – can be amplified via polymerase chain reactions (PCR)[33,34].
96
97   Batch reactions are the simplest method by which IVTT reactions can be performed, requiring a
98   single reaction vessel wherein all of the reaction components are combined[35]. Such reactions are
99   sufficient for protein expression and basic circuit testing yet prove insufficient when attempting
100 to study the long-term dynamic behavior of a network. Over the course of a batch reaction,
101 reagents are either depleted or undergo degradation resulting in a continuous decrease of the
102 transcription and translation rates. Furthermore, as reactions progress by-products accumulate
103 that can interfere with – or completely inhibit – the correct functioning of the network.
104 Ultimately, the use of batch reactors limits the dynamic behavior which can be observed, with
105 negative regulation being particularly challenging to implement[5,36].
106
107 The versatility of IVTT systems enables multiple alternative methods by which prolonged IVTT
108 reactions can be performed, ranging from continuous flow to droplet based methods as well as
109 simpler dialysis approaches[2,30,37–40]. The application of microfluidic devices offers users increased
110 control over their reactions whilst increasing the throughput and minimizing costs[35,41,42], with
111 each specific approach having its own advantages. The use of continuous flow can be easily
112 optimized for increasing expression yields however, the inability to effectively remove specific
113 reaction products makes the study of dynamic behavior non-trivial[39]. Whilst employing droplet
114 based microfluidic systems allows high-throughput screening of novel networks, the difficulty of
115 supplying fresh reagents to the reaction results in the droplets resembling small volume batch
116 reactions[43]. Dialysis based reactors allow the introduction of fresh reagents as well as the removal
117 of some reaction products however, RNA molecules and larger proteins accumulate within the
118 reactor, being too large to diffuse through the membrane pores. Furthermore, large volumes of
119 reagents are required to sustain these reactions for prolonged periods[30,44]. In 2013, Maerkl et al.
120 presented a multi-layered microfluidic device designed specifically for conducting prolonged IVTT
121 reactions[36,45]. The use of multi-layered microfluidic devices permits direct control over fluid flow,
122 allowing for the redirection of flow as well as the isolation of fluid in specific regions of the
123 device[46,47]. These isolated regions can function as independent nanoliter-scale reaction chambers
124 wherein IVTT reactions can be performed. Over the course of a single IVTT reaction, periodic
125 injections of fresh reagents into the reactor are used to replenish IVTT components and DNA
126 templates. Simultaneously, an equal volume of the old reaction solution is displaced, removing
127 reaction products. In this manner, an out-of-equilibrium environment is maintained where the
128 basal transcription and translation rates remain in steady-state, prolonging the lifetime of IVTT
129 reactions and allowing rich dynamic behaviors to occur. By applying this approach, researchers
130 are able to investigate the kinetic rates of the individual processes occurring within a specific
131 circuit, aiding in the forward-engineering of novel genetic networks. For instance,
132 Niederholtmeyer et al. implemented this approach to characterize various elements of a genetic

133 ring oscillator, determining the kinetic rates thereof[36]. In further studies, Yelleswarapu et al.
134 showed that the kinetic rates of sigma factor 28 (σ_{28}) determined under batch conditions were
135 insufficient to describe the behavior of a σ_{28}-based oscillator, and that the addition of flow-based
136 data improved model predictions of the network behavior[22].
137
138 The goal of this manuscript is to present a complete protocol for the fabrication of multilayer
139 microfluidic devices capable of performing long-term IVTT reactions. In addition, this manuscript
140 will describe all of the hardware and software required to perform prolonged IVTT reactions. The
141 actuation of the microfluidic device - necessary to control the flow of fluids therein - is achieved
142 using a series of pneumatic valves which connect directly to the microfluidic devices via lengths
143 of tubing. In turn, the pneumatic valves are controlled via a custom-built virtual control interface.
144 Fluid flow within the microfluidic devices is achieved using continuous pressure which is provided
145 by a commercially available pressure regulation system. IVTT reactions are typically performed
146 between 29 °C and 37 °C and a microscope incubator is used to regulate the temperature during
147 reactions. However, the functionality of the IVTT mixture gradually degrades when stored above
148 4 °C. As such, this manuscript will expand on the off-chip cooling system used to cool the IVTT
149 mixture prior to injection into the microfluidic device. In conclusion, this manuscript provides a
150 comprehensive overview of the procedures required to successfully perform prolonged IVTT
151 reactions using a microfluidic flow reactor such that other researchers will be able to replicate
152 this technology with relative ease.
153
154 **PROTOCOLS**
155
156 **1.   Wafer preparation**
157
158 NOTE: Our protocols are specific for the 40 XT positive photoresist and SU8 3050 negative
159 photoresist used during this research. Alternative photoresists can be used, however the specific
160 spin speeds, baking temperatures, and baking times will vary. The microfluidic device design
161 provided by Niederholtmeyer et al.[36] is linked in the **Table of Materials**.
162
163 1.1   Place two clean silicon wafers (100 mm diameter, <1-0-0> oriented, 525 µm thickness) in
164 a preheated oven set to 250 °C and leave the wafers to dehydrate overnight (~16 h).
165
166 NOTE: It is also possible to prime the wafers using HMDS vapor deposition; however, this is not
167 necessary if the wafers are sufficiently dehydrated.
168
169 1.2   Remove one silicon wafer from the oven and let it cool to room temperature before
170 proceeding with the spin coating. Apply 3-4 mL of the 40 XT photoresist to the center of the
171 wafer.
172
173 1.3   To obtain a feature height of 25 µm apply the following spin protocol: spin for 20 s at 500
174 rpm (110 rpm/s), increase the spin speed to 3100 rpm (300 rpm/s) and hold here for 30 s, and
175 decelerate the wafer to 0 rpm with a deceleration of 200 rpm/s. Using a microfiber tissue
176 carefully remove any edge beading which may have occurred during the spin coating.

177

1.4   Soft bake using two separate hot plates set to 70 °C and 120 °C in the following manner: allow the wafer to sit at 70 °C for 30 s. Then transfer the wafer to the 120 °C hotplate and allow it to rest here for 3.5 min before returning the wafer to the 70 °C hotplate for another 30 s. Remove the wafer from the hotplate and – avoiding sudden temperature shocks – allow it to cool to room temperature.

1.5   Place the flow layer photomask (emulsion side down) onto the photoresist film and expose the wafer using a UV lamp until a total exposure of 200 mJ/cm^2 is achieved.

1.6   Post-exposure bake using two hot plates (70 °C and 105 °C) in the following manner: allow the wafer to sit at 70 °C for 20 s before transferring the wafer to the 105 °C hotplate and leaving it here for 40 s. Finally return the wafer to the 70 °C hotplate for a further 20 s to complete the post-exposure bake.

1.7   Allow the wafer to cool to room temperature on a stack of microfiber tissues. Develop the wafer by transferring it to a Petri dish filled with 726 MIF photoresist developer to start the development process. Development is accelerated when performed on a benchtop shaker and the entire wafer is submerged in the developer.

1.8   Rinse the wafer with demineralized water and use a stereo microscope to check the wafer surface for any photoresist residue. If photoresist residue can be seen, then return the wafer to the developer.

1.9   Reflow the positive photoresist by placing the wafer on a hot plate set at 110 °C for 25 min. This process will result in rounded features as well as annealing any cracks that may have appeared during the fabrication process. Proceed to silanize the wafer as described in step 1.17.

1.10 Remove the second silicon wafer from the oven, allowing it to cool to room temperature before proceeding with the spin coating. Apply 5 mL of SU8 3050 photoresist to the center of the wafer.

1.11 To obtain a feature height of 30 µm apply the following spin protocol: spin for 20 s at 500 rpm (110 rpm/s), increase the spin speed to 4,000 rpm (330 rpm/s) and hold here for 42 s, and decelerate the wafer to 0 rpm with a deceleration of 200 rpm/s. Using a microfiber tissue carefully remove any edge beading which may have occurred during the spin coating.

1.12   Soft bake using two separate hot plates (65 °C and 95 °C) in the following manner: allow the wafer to sit at 65 °C for 30 s. Then transfer the wafer to the 95 °C hotplate and allow it to rest here for 14 min before returning the wafer to the 65 °C hotplate for another 30 s. Remove the wafer from the hotplate and allow it to cool to room temperature.

1.13   Measure the intensity of the UV lamp before exposure and use this to determine the exposure duration required to achieve a total exposure dosage of 260 mJ/cm^2. Place the

221 photomask (emulsion side down) onto the photoresist film and place the wafer underneath the
222 UV light source. Expose the wafer using a UV lamp until a total exposure of 260 mJ/cm^2 is
223 achieved.
224
225 1.14   Post-exposure bake using two hot plates (65 °C and 95 °C) in the following manner: allow
226 the wafer to sit at 65 °C for 60 s before transferring the wafer to the 95 °C hotplate and leaving
227 it here for 4.5 min. Return the wafer to the 65 °C hotplate for a further 30 s to complete the post-
228 exposure bake.
229
230 1.15   Allow the wafer to cool to room temperature on a stack of microfiber tissues. Develop
231 the wafer by transferring it to a Petri dish filled with mrDev-600 photodeveloper to start the
232 development process. Development is accelerated when performed on a benchtop shaker and
233 the entire wafer is submerged in the developer.
234
235 1.16   Rinse the wafer with isopropanol and use a stereo microscope to check the wafer surface
236 for any photoresist residue. If photoresist residue can be seen, then return the wafer to the
237 developer. Once fully developed, hard bake the photoresist by placing the wafer on a hot plate
238 set at 150 °C for 1 h.
239
240 1.17   Silanize both wafers to prevent the adhesion of PDMS during the soft-lithography
241 processes. To perform the silanization, pipet 2-3 droplets (per wafer) of the silane into a small
242 glass vial. Place this vial, along with the wafers into a desiccator and pull vacuum for 5-10 min.
243 Seal the desiccator and leave the wafers under vacuum for a period of 12 – 16 h.
244
245 CAUTION: The silane is toxic and should not be inhaled. Take care to work in a fume hood and to
246 wear nitrile gloves when handling the silane. This includes placing the vacuum pump in the fume
247 hood when pulling vacuum on the desiccator.
248
249 1.18   Release the vacuum from the desiccator and remove the silanized wafers. Rinse with
250 water and use a steam of N$_2$ to dry the wafers. At this point the wafers can be placed in storage
251 until required.
252
253 **2   Microfluidic device fabrication**
254
255 NOTE: The soft-lithography process used to fabricate PDMS based multilayer microfluidic devices
256 can be separated into three distinct steps: 1) The PDMS preparation of both the flow and control
257 layers, 2) The alignment and bonding of the two PDMS layers, 3) The completion of the device.
258
259 2.1   PDMS preparation
260
261 2.1.1   Prepare two PDMS precursor solutions by combining the base and curing agents in a
262 plastic beaker and using a mixing rod to stir the two components until fully mixed. The control
263 layer requires 20 g of base agent and 1 g of curing agent (20:1 ratio). The flow layer requires 40
264 g of the base agent and 8 g of the curing agent (5:1 ratio). Degas the solutions in a desiccator.

265

266 2.1.2   Place the flow layer wafer in a Petri dish and pour the 5:1 ratio PDMS mixture over the
267 wafer. Degas the PDMS for 30 min to remove air bubbles.

268

269 2.1.3   Spin coat the control layer wafer (prepared using the negative SU8 3050photoresist) with
270 20:1 ratio PDMS. Pour 5-10 mL of the PDMS onto the center of the wafer and run the following
271 spin protocol (retain the left over PDMS for later use): spin at 500 rpm for 15 s (100rpm/s),
272 increase the spin speed to 1450 rpm (300 rpm/s) for 45 s, and then decelerate the wafer to 0 rpm
273 (200 rpm/s).

274

275 2.1.4   To ensure a homogeneous PDMS film thickness, place the PDMS coated wafer on a level
276 surface in a closed Petri dish (to avoid dust contamination). Let the wafer sit for 30 min.

277

278 2.1.5   Remove the flow layer from the desiccator and place both the flow and control layers in
279 an oven (80 °C). Cure both layers for 28-30 min and remove when the PDMS is malleable enough
280 to manipulate, whilst remaining slightly sticky. Immediately proceed with the alignment process.

281

282 2.2   Alignment and bonding

283

284 2.2.1   Using a scalpel, remove each of the four devices from the PDMS on the flow layer wafer.
285 Upon removing the PDMS layer from the silicon wafer, immediately cover the feature side with
286 scotch tape to avoid dust particle contamination.

287

288 2.2.2   Roughly align the flow layer blocks on the control layer by eye, placing the feature side
289 of the device into contact with the control layer PDMS. Subsequently, make fine adjustments to
290 the position of each of the flow layer blocks to align the channels of the flow layer with the control
291 layer channels, using a stereo microscope to aid in the visualization of the adjustments.

292

293 2.2.3   Apply pressure to remove air pockets between the two PDMS layers. Pour the remaining
294 20:1 ratio PDMS saved earlier around the aligned flow layer blocks. Place 100 g weights on each
295 of the devices to ensure sufficient contact during the bonding process.

296

297 2.2.4   Return the aligned devices (including the weights) to the 80 °C oven and leave them to
298 bond for at least 1.5 h and no longer than 6 h.

299

300 2.3   Finishing the Device

301

302 2.3.1   Remove the wafer from the oven and extract each of the individual devices from the
303 control layer wafer, covering the feature side of each device with scotch tape.

304

305 2.3.2   Iteratively, punch a single hole for each of the 9 flow layer inlets, 24 control layer channel
306 inlets, and the single flow layer outlet of each device. Punch the device with the feature side
307 facing up, using a camera to ensure holes are punched within the feature boundaries.

308

309 2.3.3   For each device, clean a single microscope slide with isopropanol and acetone and dry the
310 slides under a stream of N_2. Subsequently, place the microscope slides on a hot plate set to 150
311 °C for 15 min.
312
313 2.3.4   Use oxygen plasma ashing to bond the PDMS devices to the glass slides, applying an
314 ashing power of 50 W for 45 s. Ensure that the feature side of the device is facing upwards when
315 ashing. Once complete, place the device feature side down on the glass slide, applying pressure
316 to remove trapped gas between the surfaces.
317
318 2.3.5   Place the bonded devices onto a hot plate set to 110 °C for 1 h. Weights can be placed on
319 top of the devices to improve adhesion of the device.
320
321 **3   Hardware setup**
322
323 NOTE: To achieve control over the microfluidic chips, numerous pieces of hardware need to be
324 installed and connected with one another. Three distinct groups of hardware are required: 1)
325 Pneumatic control system for the control channels, 2) A pneumatic pressure regulator to control
326 flow of the reaction reagents within the device, and 3) A cooling system to cool the IVTT reaction
327 solution prior to injection into the microfluidic device. An overview of the hardware setup is
328 provided in **Figure 1.** It should be noted that the protocols provided here attempt to be as general
329 as possible, however certain specific pieces of equipment used throughout our research are
330 referenced. All hardware can be replaced by alternatives able to perform the same function. In
331 such cases, the protocols here can be used to outline the general steps needed to set up the
332 system and the requirements of each of the components. Alternative hardware setups are
333 presented by Brower et al.[48] and White and Streets[49].
334
335 3.1   Pneumatic control system (see **Figure 2**)
336
337 3.1.1   Using the manufacturers' protocol, establish a TCP connection between the fieldbus
338 controller and the user workstation. Use control software (provided as supplementary files) to
339 compose MODBUS commands which are sent via the TCP connection to controller, and actuate
340 the solenoid valves.
341
342 3.1.2   Expand the fieldbus controller with eight 4-channel digital output modules, one for each
343 solenoid valve being used. Each solenoid valve presides over a connecting pin. Connect the
344 positive wire to one of the four positive outputs on one of the digital output modules whilst
345 connecting the negative wire to one of the ground ports of the digital output modules.
346
347 NOTE: To get the system to work correctly, the solenoids should be connected systematically,
348 with the first solenoid being connected to the first output port, the second solenoid to the second
349 output port and so on. In our system, two valve arrays are used with 8 and 22 solenoid valves
350 respectively. Output ports 1-8 connect to the 8-valve array, and output ports 9-30 connect to the
351 22-valve array.
352

353 3.1.3   Connect both valve arrays to a compressed air source using 1/4" tubing. Use pressure
354 regulators to set the pressure of the 22-valve array to 3 bar, and the 8-valve array to 1 bar.
355
356 3.2   Flow pressure regulation (see **Figure 3**)
357
358 NOTE: To flow fluids through the flow layer of the microfluidic device, a commercially available
359 4-port pressure regulator is used. The output pressure of each port is regulated via software
360 provided with the pressure controller. Connect the pressure regulator to a computer using the
361 supplied USB-connector.
362
363 3.2.1   Connect the pressure regulator to a compressed air source, ensuring that the supplied
364 pressure does not exceed the maximum pressure permitted by the regulator.
365
366 3.2.2   Connect a male Luer to 3/32" barb connector to each of the four female Luer lock output
367 ports of the pressure regulator. Connect a length of soft tubing (OD: 3 mm, ID: 1 mm, L: 10 cm)
368 to the barb.
369
370 3.2.3   Connect a second male Luer to 3/32" barb connector to the open end of the soft tubing
371 and attach this to the fluid reservoir connector ports.
372
373 3.2.4   Use the provided software to set the desired pressure of each outlet of the flow regulator,
374 pressurising the reagents stored within the reservoirs to result in the flow of the reagents into
375 the microfluidic device. The connection of the reservoirs to the microfluidic device will be
376 discussed in the section 4.2.
377
378 3.3   Off-Chip cooling setup (see **Figure 4**)
379
380 3.3.1   Use PVC tubing (OD: 10 mm, ID: 6 mm) to connect the water-cooling system to the cold-
381 plate water block using compression fittings. Fill the fluid reservoir of the water-cooling system
382 with a coolant and gently tilt the unit to displace any trapped air, continuously adding coolant to
383 the reservoir to ensure it remains full. When all the gas is removed from the system, fill the
384 reservoir to approximately 90-95% of its maximal volume.
385
386 3.3.2   Coil PTFE tubing (OD: 0.042", ID: 0.022") onto the cold face of the Peltier element and
387 secure this with tape. Ensure that one end of the PTFE tubing is connected to the reservoirs of
388 the flow layer pressure control system (as described in section 4.3). The other end of the PTFE
389 tubing should protrude no more than 1 cm from the Peltier surface. Insert a 5 – 10 cm length of
390 PEEK tubing (OD: 0.794 mm, ID: 0.127 mm) into the protruding end of the PTFE tubing. The filling
391 of the tubing and connection to the microfluidic device is further explained in section 4.3.
392
393 3.3.3   Place the hot face of the Peltier element onto the cold-plate of the water block, applying
394 sufficient thermal compound to the two faces. Ensure that the tubing, Peltier element, and
395 cooling block are in direct contact with one another at all times.
396

397 3.3.4   Connect the Peltier element to the temperature controller (via a serial bus connector),
398 such that the voltage supplied to the Peltier can be regulated. Securely place a thermistor on the
399 Peltier surface, connecting the output to the temperature controller. After turning on the water
400 cooler, adapt the voltage supplied to the Peltier until the temperature is stable at 4 °C.

402 NOTE: With this setup, the Peltier temperature is controlled manually by adapting the supplied
403 voltage, while the thermistor serves only to monitor the temperature.

405 **4**   **Preparing an experiment**

407 NOTE: Prior to starting an experiment, the microfluidic device must be prepared, and the reaction
408 reagents must be inserted into the correct tubing for injection into the device. This section will
409 discuss: 1) The connection of control channel tubing to the device, 2) The connection of uncooled
410 inflow reagents to the device, and 3) The connection of cooled inflow reagents to the device.

412 **4.1**   Connecting the control channel tubing

414 4.1.1   For each of the control channels of the microfluidic device, cut a length of tubing (OD:
415 0.06", ID: 0.02"). At one end, insert the pin of a 23 G, 1/2" Luer stub and at the other insert a
416 stainless steel connecting pin (OD: 0.65 mm, ID: 0.35 mm, L: 8 mm).

418 4.1.2   Connect the Luer stub to a male Luer to 3/32" barb nylon connector. Insert the barb of
419 the connector into a length of polyurethane tubing (OD: 4 mm, ID: 2.5 mm). Insert this
420 polyurethane tubing directly into one of the solenoid valves.

422 4.1.3   Attach a 23 G, 1/2" Luer stub to a syringe and insert this into a short (3-4 cm) piece of
423 tubing (OD: 0.06", ID: 0.02"). Place the open end of this tubing into a reservoir of ultrapure water
424 and fill the syringe with ultrapure water.

426 4.1.4   Number each control channel of the microfluidic device as shown in **Figure 5**. For each
427 channel (excluding control channels 1 through 3, which are not filled with water), find the
428 corresponding tubing (connected to the solenoid valves) and insert a metal pin into the open end
429 of the tubing attached to the syringe. Inject water into the control channel tubing until half the
430 length has been filled.

432 4.1.5   Disconnect the tubing from the syringe and insert the stainless-steel connector pin into
433 the corresponding hole of the microfluidic device. Repeat for all control channels.

435 4.1.6   Use the control interface to open all the solenoid valves. This will pressurize the fluid
436 within the control channel tubing, forcing it into the microfluidic device and closing all the
437 membrane based valves within the device. An example of open and closed membranes within
438 the device are given in **Figure 6.**

440 **4.**2   Connecting uncooled reagents to the device

4.2.1 For each of the uncooled reagents, cut a length of tubing (OD: 0.06", ID: 0.02") to connect the reservoir outlet to the microfluidic device inlets.

4.2.2   Take one end of the tubing and insert this into the reservoir, ensuring that the tubing reaches the base of the reservoir. The reservoir tubing outlet should be tightened such that an air tight seal is achieved. Insert a stainless-steel connection pin (OD: 0.65 mm, ID: 0.35 mm, L: 8 mm) into the open end of the tubing.

4.2.3   Attach a 23 G, 1/2" Luer stub to the end of a small (1 mL) syringe. Add a short length of tubing (OD: 0.06", ID: 0.02") to the Luer stub. Placing the end of the tubing into the desired reagent solution, fill the syringe with the reagent.

4.2.4   Insert the stainless-steel connector pin into the polyurethane tubing connected to the syringe and fill the tubing with the reagent. When using small reaction volumes, the reagent will not enter the reservoir, and the tubing itself will act as the reservoir. Disconnect the syringe and insert the connector pin into one of the flow layer inlet holes of the microfluidic device.

4.2.5   Apply pressure to each of the reservoirs using the pressure regulator software to force the reagents into the microfluidic device.

4.3   Connecting cooled fluids to the microfluidic device

4.3.1   Ensure that the water cooler and Peltier element have been turned on, with the surface temperature of the Peltier set to 4 °C. Mount the cooling setup as close to the microfluidic device as possible, minimising the uncooled volume between the Peltier and the device inlet.

4.3.2   Connect the open end of the PTFE tubing to tubing connected to one of the fluid reservoirs (as described in section 4.2) using a stainless-steel connector pin (OD: 0.65 mm, ID: 0.35 mm, L: 8 mm).

4.3.3   Connect a small syringe (1 mL) to a Luer stub (23 G, 1/2") with a short length of tubing (OD: 0.06", ID: 0.02") attached to the end. Fill the syringe with the to-be-cooled reagent (here, the IVTT reaction solution).

4.3.4   Connect the PEEK tubing to the syringe via the connective tubing and apply constant pressure to the syringe, forcing the reagent through the PEEK tubing and into the PTFE tubing. Disconnect the PEEK tubing from the syringe and insert it directly into one of the flow channel inlets of the microfluidic device. When pressure is applied via the pressure regulator software the cooled reagent will be forced into the microfluidic device.

5   Experimentation

484 NOTE: Prior to performing experiments all the hardware and tubing connections detailed in
485 protocols sections 3 and 4 should be completed, and all the reagents should be connected to the
486 device. The experimental procedure can then be divided into four distinct parts: 1) The loading
487 of the microfluidic device, 2) Preparing the microscope, 3) The calibration of the device, and 4)
488 Performing the experiment. The custom virtual control interface (see **Figure 7**) used throughout
489 this research is provided as a supplementary resource via the Materials list)
490
491 **5.1   Loading the microfluidic device**
492
493 5.1.1   Place the microfluidic device, with all control and flow layer tubing attached into the
494 microscope stage and close any openings on the incubator. Set the ambient temperature of the
495 incubator to 29 °C. Ensure that the cooling system has been turned on and is set to 4 °C prior to
496 initiating the experiment.
497
498 5.1.2   Ensure all the flow and control channels are pressurized. Set the pressure of control
499 channels 1-3 at 1 bar, and pressurize control channels 9-29 at 3 bar. The reagents require a
500 pressure of between 20 and 100 mbar to be applied to the fluid reservoirs using the pressure
501 regulator software.
502
503 5.1.3   Remove air from the microfluidic device using one of the reagents. Close the outlet of the
504 device (pressurize control channel 29) and simultaneously depressurize control channels 1-3, and
505 15-28. Then selectively depressurize the control channels of the multiplexer to allow the selected
506 reagent to flow into the device. Use the microscope to monitor the removal of air.
507
508 NOTE: In the case that reagents are not loading into the device correctly, or that the air bubbles
509 are not being removed, the pressure can be increased up to 350 mbar.
510
511 5.1.4   Ensure that all reagents flow correctly, without introducing air - using the **Flush** function
512 in the control software. Monitoring of the fluid flow can be simplified by first loading a
513 fluorophore and monitoring its displacement by individual reagents.
514
515 **5.2**   Preparing the microscope
516
517 5.2.1   Using the microscope, locate any points of interest (a single point within each reactor is
518 sufficient) within the microfluidic device, and store the coordinates thereof. These points will be
519 imaged during the calibration and experimental processes.
520
521 NOTE: During the calibration and experimental procedures included within the control software,
522 the microscope is instructed to periodically capture images at the previously stored coordinates.
523 To achieve this, the control software communicates with the microscope software, informing it
524 to record new images. This communication is unique to each microscope setup, and as such this
525 functionality has been modified within the provided software interface. Provided is a dummy
526 executable, which can be modified by the end-user for compatibility with their own microscope
527 system.

5.3   Calibrating the microfluidic device

5.3.1   Determine the fluid volume displaced from each reactor during a single inflow step (pump sequence provided by peristaltically actuating control channels 15-17, comprising 6 MODBUS commands executed sequentially), by executing the calibration protocol provided in the software package.

5.3.2   Set the following data fields within the control software: **Elution Buffer Channel**, **Fluorophore Channel, Number of Dilution Cycles** (default is 10 dilutions), **Number of Inflow Steps** (default is 15 steps), **Number of Mixing Cycles** (default is 4 cycles), and **Time between Mixing Cycles** (default is 0 seconds). Initiate the calibration by pressing **Perform Calibration Experiment.**

NOTE: During the calibration process, all reactors are filled with a fluorophore and images are recorded. Subsequently, a series of dilutions are performed where an eluent is metered into the reactors (based on the set number of inflow steps), thus displacing the fluorophore. After thoroughly mixing, new images are taken. This process repeats for the set **Number of Dilution Cycles**.

5.3.3   Upon completion of the calibration, follow the steps presented by the control software, completing the analysis of the calibration experiment.

NOTE: The analysis will provide users with the **Refresh Ratio** for each reactor based on the fluorescence decrease recorded during each **Dilution Cycle**. This value indicates the fraction of the reactor volume displaced by the set **Number of Inflow Steps**. In turn, this value will be used during experiments to determine how many inflow steps are required to displace a specific reactor volume.

5.4   Performing the Experiment

5.4.1   Set the required values for the desired experiment within the virtual control interface. Critical is the **Refresh Fraction [%]** which determines the reactor volume displaced per experimental cycle and should be set between 15 and 40%.

NOTE: The specific experimental protocol will determine which fields of the control interface should be set prior to the initiating the experiment. Some minor coding will be required to adapt the control interface for novel experiments.

5.4.2   Initiate the experimental protocol by pressing the **Perform Experiment** button in the control interface.

NOTE: Unaltered, the provided software initiates a simple protein expression. Reactors 1 and 8 are utilized as controls, while reactors 2-7 house identical experiments. Here, 75% of the reactor

volume comprises IVTT solution, and 25% is either ultrapure water or a 2.5 nM linear DNA solution. Dilutions occur every 15 minutes, with 30% of the reactor volume being displaced per dilution. Images are recorded at the end of each dilution cycle.

6   Data analysis

NOTE: Scripts have been provided for the analysis of the images (see supplementary files or the **Table of Materials**), making use of the *'bfopen'* analysis package, which is required for the reviewing of '*.nd2*' files (provided by our microscope setup).

6.1   Run the analysis script '*calibrationScript.m*' and once prompted select the desired '*.nd2*' file. A single reactor image will be shown with which the correct image intensity can be determined. Use the slider to optimize the image intensity such that the edges of the microfluidic channel are clearly visible.

6.2   An image of the reactor will be shown. Within this image, select an area inside the reactor channel of which the fluorescence intensity should be determined.

NOTE: The fluorescence intensity of each reactor, for each recorded image will be determined with the results displayed in a simple plot, allowing the visualisation of the results.

REPRESENTATIVE RESULTS
To demonstrate the effectiveness of the multilayer microfluidic platform for the conduction of IVTT experiments, the described setup was used to express the deGFP protein. The experiment was conducted in a commercially available[30] IVTT reaction mixture – comprising all the necessary transcription and translation componentry – supplemented with reaction substrates and DNA templates. Experiments were conducted at a temperature of 29 °C; a temperature found to be optimal for the IVTT expression of proteins.

The microfluidic device possesses nine unique inlets, of which four were utilized during this experiment. The first contained the commercially obtained IVTT reaction mixture. The IVTT reaction mixture accommodates all the components required to successfully express proteins however, purified GamS was added to the reaction mixture – at a final concentration of 1.3 µM - prior to loading into the microfluidic device. The addition of the GamS protein serves to minimize the degradation of linear DNA species when performing the experiments. Crucially, the IVTT mixture was injected into polytetrafluoroethylene (PTFE) tubing coiled onto a Peltier element with a surface temperature of 4 °C to cool the solution prior to the injection thereof into the microfluidic device; preventing the degradation of the reaction solution prior to its use. Micro-bore polyether ether ketone (PEEK) tubing was used to connect the PTFE tubing leaving the Peltier element surface with the microfluidic device, reducing the volume of the IVTT reaction mixture not being cooled. The second solution inserted into the device contained the linear DNA template coding for the deGFP – dissolved in ultrapure water – at a concentration of 10 nM. The third solution, ultrapure water, served multiple purposes during the experimental procedures. Primarily, the ultrapure water was used to ensure that the displaced volume per dilution was

equal for all reactors, acting as a replacement for DNA in the control reactions. Additionally, ultrapure water was also used to dilute the fluorophore during the device calibration and to flush the dead volume of the device when switching between reagents. The final solution inserted into the device was a purified FITC-dextran solution (25 µM) required to perform the initial device calibration. The DNA, water, and fluorophore solutions were injected into tubing (0.02" ID, 0.06" OD) which could subsequently be inserted into one of the inflow channels of the microfluidic device as per Section 4.2 of the protocols. As such, these solutions were stored at 29 °C for the entirety of the experiments.

The actuation of the control channels of the microfluidic device is achieved via custom control software where each of the control channels can be individually actuated. The execution of prolonged IVTT reactions cannot be achieved via this manual process and requires the use of automated protocols incorporated within the control software. When preparing a microfluidic device for experiments, similar automated protocols can be utilized to execute a number of useful processes: the flushing of the device dead volume with a new reagent, the mixing of the reagents within the ring reactor, and the loading of a new reagent into the reactor whilst displacing an equal volume of the current solution. In addition, two complex process are available: the conduction of a device calibration, and the execution of a prolonged cell-free protein expression. All of the aforementioned processes can be easily executed from the main interface, alongside the ability to configure multiple parameters to vary specific process settings such as the inflow channel, inflow volume, and mixing duration.

Due to fluctuations in pressure and imperfections during microfluidic device fabrication, the volume of fluid displaced during a single injection cycle can vary between devices. As such, prior to performing IVTT experiments, the displaced reactor volume per injection cycle (**Refresh Fraction**) was determined. This calibration requires the filling of all eight reactors with a fluorescent reference solution. In this case, a purified FITC-dextran solution (25 µM) was used. Subsequently, the reactors are diluted 10 times with ultrapure water. By measuring the decrease in fluorescence per dilution cycle for each reactor, the volume of fluid displaced during a single injection cycle was determined. Within the control software, this value (the **Refresh Ratio**) was recorded for use during the IVTT experiment. Crucially, to account for variations in the flow rate across the device, as well as discrepancies in the individual reactor volumes, the **Refresh Ratio** is determined and stored for each individual reactor. The sequence of filling and diluting the reactors was conducted automatically using the **Perform Calibration** program which forms part of the control software. The results of the calibration experiment are shown in **Figure 8.**

The most complex pre-programmed process executes a long-duration IVTT experiment, allowing users to initiate the experiment and subsequently allow it to operate unattended until completion. Throughout the experiment, reactors 1 and 5 were used as blanks, with only water being added to the reactors during dilutions. Reactors 2 and 6 were utilized as negative controls and contained only IVTT reaction solution and ultrapure water. The remaining reactors (3, 4, 7, and 8) contained the IVTT reaction solutions and 2.5 nM of linear DNA coding for the deGFP gene. Initialisation of the reactors is achieved by fully filling all the reactors (excluding 1 and 5) with the IVTT reaction solution, before 25% of the reactor volume was displaced with ultrapure water.

660   Hereafter, the periodic injection of reagents into the reactors was initiated. The experiment was
661   conducted such that new reagents were injected into the reactors every 14.7 minutes, with 30%
662   of the reactor volume being displaced during each dilution cycle. The composition of each
663   injection was such that 75% of the injected fluid comprised fresh IVTT solution, whilst the
664   remaining 25% consisted of either DNA or ultrapure water. Following each injection of new
665   reagents the reactors were continuously mixed, after which a fluorescence image of each reactor
666   was recorded using the microscope. The reaction was subsequently allowed to run continuously
667   for 68 cycles, resulting in an experimental duration of 16.5 h. The results of this experiment are
668   given in **Figure 9.**

669

670   When performing prolonged IVTT experiments, there are two main causes for the failure of a
671   reaction; the introduction of air into the microfluidic device or the degradation of the IVTT
672   reaction solution. The occurrence of air within the microfluidic device is most often the direct
673   result of small air bubbles existing in the inflow solutions, which are subsequently injected into
674   the microfluidic device. Upon entering the device, the presence of air inhibits the proper flow of
675   fluids, whereby the reactions are no longer periodically refreshed leading to the formation of
676   batch reactions within the reactor rings. In some cases, the air is slowly removed from the device
677   by the repeated flushing of reagents, after which the reaction continues as intended (as shown
678   in **Figure 9**). In other cases the air remains trapped, and can only be removed by aborting the
679   experiment and subsequently applying continuous (high) pressure to the flow layer of the
680   microfluidic device, analogous to the filling process described in Section 5.1 of the protocols.
681   During our experiments the cell lysate is stored in PTFE tubing on a Peltier element cooled to 4
682   °C. Both measures aid in limiting the degradation of the IVTT reaction solution over time, with
683   the inert PTFE tubing ensuring limited interaction between the tubing and the reaction solution
684   and the cold temperatures preserving the functional (bio)molecular componentry required to
685   perform IVTT. Should degradation of the reaction solution occur – as the result of insufficient
686   cooling or undesired interactions between the reaction solution and the storage environment –
687   then this will exhibit itself experimentally as a gradual reduction of protein expression over time.
688   Once degraded, the IVTT reaction solution cannot be recovered and a new experiment should be
689   prepared.

690

691   **FIGURE LEGENDS:**
692   **Figure 1. The hardware setup required to perform continuous IVTT reactions. A**) Schematic of
693   the hardware setup. **B**) Photograph of the setup used throughout this manuscript. The
694   implementation of a multilayer microfluidic device for continuous IVTT reactions requires an
695   extensive hardware setup to regulate flow pressure, actuate control channels, heat and cool
696   reactions and reagents, store fluids, and image the device during experiments. Experiments are
697   performed at temperatures of 30 °C, which is achieved by placing the microscope within an
698   incubator set to this temperature. To prevent deterioration of the IVTT reaction solution, it is
699   stored within PTFE tubing coiled over the cold face of a Peltier element. The temperature of the
700   Peltier element is set to 4 °C, with a water cooler and water block being used to maintain this
701   temperature. Reagents which do not require cooling, are stored in fluid reservoirs outside of the
702   microscope incubator. Constant pressure is applied to these reservoirs by a computer controlled
703   pressure regulator. In this manner, the fluids are forced through the outlet tubing of the

704 reservoirs, which connect directly to the inflow channels of the microfluidic device. Each of the
705 control channels of the microfluidic device is connected to a pneumatic valve. The entire valve
706 array is under constant pressure. Opening the valve, allows for pressurisation of the fluid within
707 the tubing connecting the pneumatic valve to the control channel of the microfluidic device, thus
708 opening and closing the PDMS membranes found within the microfluidic device. The pneumatic
709 valves are opened and closed via a user interface which commands a fieldbus controller (not
710 shown) to open and close specific pneumatic valves. Figure adapted from Yelleswarapu et al.[22].
711
712 **Figure 2. Overview of the pneumatic valve setup and control channel connection.** An 8-valve
713 array is shown with three control channel connections fitted to valves 1, 2, and 3. Compressed air
714 can be supplied to the valve array via 1/4" tubing. For the actuations of control channels two
715 pressures are used: 1 bar for the lower pressure control channels (1, 2, and 3) and 3 bar for the
716 higher pressure control channels (9 through 30, not shown here). The tubing can be filled with
717 ultrapure water and inserted into one of the control channel inlets using a stainless steel
718 connector pin.
719
720 **Figure 3. Overview of the commercial flow pressure regulator and reservoir system.** A
721 commercially available pressure regulator is used to inject fluids into the flow layer of the
722 multilayer microfluidic device. Connecting the pressure controller to a computer allows for
723 modulation of the pressure used to perform the fluid injections. Reagents can be stored in a fluid
724 reservoir, which is directly connected to the pressure regulator. The application of pressure to the
725 reservoir forces the fluid out of the reservoir via the outlet tubing. This outlet tubing can be
726 connected directly to one of the fluid inlets of the microfluidic device using a stainless steel
727 connector pin. In the event that the reagent volume is unable to reach the fluid reservoir, the
728 outlet tubing acts as a reservoir for the reagent.
729
730 **Figure 4. Overview of the cooling system used to cool reaction reagents. (**Left) Isolated cooling
731 setup and (Right) Cooling setup placed within the microscope and connected to the microfluidic
732 device. A Peltier element is used to cool the IVTT reaction solution prior to injection into the
733 microfluidic device. The reagent is stored within PTFE tubing coiled over the cold-face of the
734 Peltier element. A length of PEEK tubing is used to transfer the cooled fluid to the microfluidic
735 device, with the small internal diameter (0.005") minimizing the reagent volume no longer being
736 cooled. Alongside the coiled PTFE tubing, a thermistor is placed, allowing for real-time
737 temperature monitoring on the surface of the Peltier element. The voltage applied to the Peltier
738 is set such that the surface temperature of the Peltier remains between 0 °C and 4 °C. To remove
739 excess heat produced by the Peltier element, the hot-face of the Peltier is placed against a water
740 cooled block, with the addition of silicone free heat sink grease ensuring optimal heat transfer
741 between the two faces.
742
743 **Figure 5. Overview of the microfluidic device design.** The microfluidic flow reactor for continuous
744 IVTT reactions consists of eight reactor rings, each with a volume of 10.7 nL. Nine inlets allow for
745 the inflow of nine unique reaction solutions into the device. 24 control channels regulate the flow
746 of fluids within the device. Control channels 9 through 14 form a multiplexer. These control
747 channels should be pressurized at all times to inhibit fluid flow into the device. Depressurisation

748 of two control channels simultaneously allows for the inflow of a single reagent. Control channels
749 15, 16, and 17 are used to peristaltically pump the reagents into the device in a controlled manner.
750 Control channels 18 through 25 each control the inlet of one of the eight reactors found within
751 the device. Control channel 26 can close the flush channel, thus forcing fluid into the reactors.
752 Control channel 27 aids in the homogeneous filling of the reactors. Control channels 28 and 29
753 regulate the ring reactor outlets and the only device outlet respectively. Finally, control channels
754 1, 2, and 3 are used to peristaltically pump the fluid within the ring reactors, resulting in mixing
755 of the reagents. The design of this microfluidic device and the figure are both adapted from
756 Neiderholtmeyer et al.[29].

757

758 **Figure 6. Membrane based valve within the microfluidic device. A**) Flow channel within the
759 microfluidic device. Two control channels can be seen in the background. These channels are not
760 pressurized and as such the valves are open (fluid can flow). **B**) The two control channels
761 intersecting the flow layer channels have been pressurized, closing the valves (i.e., fluid flow is
762 impeded). Upon pressurization of the control channels, the thin PDMS membrane separating the
763 flow and control layer channels is deflected upwards (the control layer lies beneath the flow layer)
764 which closes the flow layer channel. The rounding of the flow layer channel is critical in ensuring
765 that the deflected membrane fully closes the flow channel.

766

767 **Figure 7. User interface used to control microfluidic device.** Throughout this research, a custom
768 control interface has been used to control the flow of fluids within the microfluidic devices. The
769 interface allows users to individually actuate each of the control channels (numbered 1-3 and 9-
770 29), or to execute elaborate protocols resulting in the flushing and loading of reagents, the
771 calibration of the microfluidic device, and the execution of experiments.

772

773 **Figure 8. Results of a calibration experiment.** During a calibration experiment, the reactors are
774 filled with a fluorophore (25 µM FITC-Dextran) after which the fluorescence intensity is recorded.
775 Subsequently, a series of dilutions follow, where a set number of inflow steps (15) are used to
776 inject ultrapure water into the reactors. After each dilution, the reagents are mixed and the
777 fluorescence is measured. The decrease in the fluorescence intensity per dilution reveals the
778 volume of the reactor ring displaced for the set number of inflow steps; a value termed the
779 **Refresh Ratio**. **A**) The average intensity and standard deviation of all eight reactors is shown in
780 red, with the individual intensity traces shown in grey. **B**) The average **Refresh Ratio** and standard
781 deviation is shown for each dilution step in red. The individual **Refresh Ratios** of each individual
782 reactor are shown in grey. It can be seen that seven of the eight reactors show very similar
783 behavior, however one reactor shows fluctuations in the **Refresh Ratio** after the seventh dilution
784 cycle. This highlights the need for unique **Refresh Ratios** for each of the reactors, as opposed to
785 using an average *Refresh Ratio* for the injection of reagents into the reactors.

786

787 **Figure 9. Results of an IVTT experiment expressing the deGFP protein.** A prolonged IVTT reaction
788 was initiated such that 30% of the reactor volume is displaced every 14.6 minutes. The reaction
789 was allowed to run for over 16 hours before being terminated. Two reactors of the microfluidic
790 device were used as blanks, with only ultrapure water being flown through the reactors
791 throughout the experiment (reactors 1 and 5). All the other reactors comprised 75% IVTT reaction

792 solution and 25% of either ultrapure water (reactors 2 and 6) or 2.5 nM linear DNA templates
793 coding for the expression of deGFP (reactors 3, 4, 7, and 8). In all four reactors where DNA was
794 added, there is clear deGFP expression. Three of the four reactors provide similar fluorescence
795 intensity, with one reactor displaying lower fluorescence signal. This could be caused by a
796 disparity in flow resulting in less DNA entering the reactor, or due to variations in the reactor
797 dimensions. After 14 hours, a sudden increase is seen in the signal of the reactors containing DNA.
798 This is caused by an air bubble entering the flow layer of the microfluidic device, presumably
799 originating from one of the inflow solutions. The trapping of air in the microfluidic device
800 significantly limits the flow of fluids through the channels, whereby no fresh reagents can be
801 added to or removed from the reactors until the air has passed. Upon resumption of flow, the
802 experiment returns to its previous fluorescence intensity.
803
804 **DISCUSSION**
805 A PDMS-based multilayer microfluidic device has been presented, and its capability to sustain
806 IVTT reactions for prolonged periods of time has been demonstrated. Although well-suited for
807 this specific example, this technology can conceivably be used for numerous other applications.
808 The additional control over fluid flow – paired with the ability to continuously replenish reaction
809 reagents whilst removing (by)products – is ideal for continuous synthesis reactions, the
810 investigation of various dynamic behaviors, and the simultaneous conduction of multiple
811 variations of a single reaction.
812
813 Despite the relatively straightforward fabrication process of PDMS based devices, the use thereof
814 requires an extensive hardware setup. Comprising valve arrays, pressure regulators, pressure
815 pumps, incubators, and cooling units, the transition from fabrication to use is not elementary,
816 and requires a significant initial investment. In addition, the ability to consistently set-up and
817 perform successful experiments with these devices requires a significant time-investment; a
818 point which this manuscript aims to address. However, once in place, the entire setup can be
819 modified for a range of purposes. Furthermore, the hardware setup comprises numerous
820 modular elements, each of which can be expanded to allow more complex microfluidic device
821 designs to be employed. Additionally, the modular design enables the replacement of hardware
822 components by similarly functioning alternatives, such that users are not limited to the specific
823 setup described here[48,49].
824
825 Variability between individual devices, and in the external conditions (such as pressure
826 fluctuations) can result in inaccuracies when performing experiments using these devices. To
827 address this issue, a calibration of the system should be performed prior to each experiment,
828 providing a unique **Refresh Ratio** for each of the reactors. Whilst the calibration addresses the
829 device-to-device and experiment-to-experiment variations, it is a time consuming process and
830 not flawless. Fluids with differing viscosities will not flow with the same rate when exposed to
831 identical pressure, and as such performing the calibration with multiple reagents may not yield
832 identical **Refresh Ratios**. This effect is attenuated by utilising three control channels to
833 peristaltically pump the reagents into the microfluidic device, as opposed to regulating the flow
834 by varying the supplied pressure only. As a last resort in cases where the disparity in viscosity is

835 very large, a unique **Refresh Ratio** can be implemented for each individual reagent by performing
836 multiple calibration experiments.

837

838 The use of a peristaltic pump to inject reagents into the microfluidic device attenuates the effects
839 of using solutions with varying viscosities, however it also creates a secondary problem. Using
840 discrete steps to pump fluids into the microfluidic device, means that the resolution of injections
841 into a single reactor, is limited by the volume injected when performing a single pump cycle.
842 Within our research this value – determined during the calibration – is approximately equal to
843 1%, indicating that a single pump cycle displaces approximately 1% of the reactor volume (about
844 0.1 nL). As such, displacing 30% of the reactor volume requires the execution of 30 pump cycles,
845 with 23 pump cycles of the IVTT reaction solution being added, and only 7 pump cycles of DNA
846 or ultrapure water being added. Although sufficient for our research, alternative experimental
847 protocols may encounter problems when attempting to add larger numbers of unique reagents,
848 use a lower **Refresh Fraction**, or add smaller volumes of a single reagent to a reactor. In such
849 cases, the microfluidic device design can be adapted to provide reactors with a larger volume. An
850 example of such is reported in Niederholtmeyer et al.[36].

851

852 Crucially, the device outlined within this manuscript allows reactions to be sustained for
853 prolonged durations resulting in steady-state transcription and translation rates. By periodically
854 injecting new reagents into the reactors – and removing reaction (by)products – the reactions
855 are sustained and complex dynamic behaviors can be monitored. In this way, a platform has been
856 created that – to some extent – mimics the cellular environment. Furthermore, this platform
857 enables the exploration of the system dynamics, by adapting the period between injections and
858 the specific composition of the injections. As a result, these multilayer microfluidic devices are a
859 powerful tool for the characterisation and optimisation of novel synthetic networks which display
860 complex dynamic behavior.

861

869

870 **DISCLOSURES**
871 The authors declare that they have no competing financial interests.

872

873 **REFERENCES**
874 1.   van Roekel, H. W. H. et al. Programmable chemical reaction networks: emulating
875   regulatory functions in living cells using a bottom-up approach. *Chemical Society Reviews*
876   **44,** 7465–7483 (2015).
877 2.   Yutaka Hori, Chaitanya Kantak, R. M. M., A. R. A. Cell-free extract based optimization of
878   biomolecular circuits with droplet microfluidics. *Lab on a Chip* (2017).

879   doi:10.1039/C7LC00552K

880 3.   Del Vecchio, D., Sontag, E. D. Dynamics and control of synthetic bio-molecular networks.
881   in *Proceedings of the American Control Conference* 1577–1588 (2007).
882   doi:10.1109/ACC.2007.4282302

883 4.   Purnick, P. E. M., Weiss, R. The second wave of synthetic biology: from modules to
884   systems. *Nature Reviews Molecular Cell Biology* **10,** 410–422 (2009).

885 5.   Padirac, A., Fujii, T., Rondelez, Y. Bottom-up construction of in vitro switchable
886   memories. *Proceedings of the National Academy of Sciences of the United States of*
887   *America* **109,** E3212–E3220 (2012).

888 6.   Guido, N. J. et al. A bottom-up approach to gene regulation. *Nature* **439,** 856–860 (2006).

889 7.   Genot, A. J., Fujii, T., Rondelez, Y. In vitro regulatory models for systems biology.
890   *Biotechnology Advances* **31,** 789–796 (2013).

891 8.   Elowitz, M. B., Leibler, S. A synthetic oscillatory network of transcriptional regulators.
892   *Nature* **403,** 335–338 (2000).

893 9.   Gardner, T. S., Cantor, C. R., Collins, J. J. Construction of a genetic toggle switch in
894   Escherichia coli. *Nature* **403,** 339–342 (2000).

895 10. Montagne, K., Plasson, R., Sakai, Y., Fujii, T., Rondelez, Y. Programming an in vitro DNA
896   oscillator using a molecular networking strategy. *Molecular Systems Biology* **7,** 466–466
897   (2014).

898 11. Khalil, A. S., Collins, J. J. Synthetic biology: applications come of age. *Nature Reviews*
899   *Genetics* **11,** 367–379 (2010).

900 12. Hockenberry, A. J., Jewett, M. C. Synthetic in vitro circuits. *Current Opinion in Chemical*
901   *Biology* **16,** 253–259 (2012).

902 13. Vecchio, D. Del. Modularity, context-dependence, and insulation in engineered biological
903   circuits. *Trends in Biotechnology* **33,** 111–119 (2015).

904 14. Cardinale, S., Arkin, A. P. Contextualizing context for synthetic biology - identifying causes
905   of failure of synthetic biological systems. *Biotechnology Journal* **7,** 856–866 (2012).

906 15. Venturelli, O. S. et al. Programming mRNA decay to modulate synthetic circuit resource
907   allocation. *Nature Communications* **8,** 1–11 (2017).

908 16. Scott, M., Mateescu, E. M., Zhang, Z., Hwa, T. Interdependence of cell growth and gene
909   expression: origins and consequences. *Science* **330,** 1099–1103 (2010).

910 17. Borkowski, O., Ceroni, F., Stan, G. B., Ellis, T. Overloaded and stressed: whole-cell
911   considerations for bacterial synthetic biology. *Current Opinion in Microbiology* **33,** 123–
912   130 (2016).

913 18. Liao, C., Blanchard, A. E., Lu, T. An integrative circuit–host modelling framework for
914   predicting synthetic gene network behaviours. *Nature Microbiology* (2017).
915   doi:10.1038/s41564-017-0022-5

916 19. Noireaux, V., Bar-Ziv, R., Libchaber, A. Principles of cell-free genetic circuit assembly.
917   *Proceedings of the National Academy of Sciences of the United States of America* **100,**
918   12672–12677 (2003).

919 20. Shimizu, Y. et al. Cell-free translation reconstituted with purified components. *Nature*
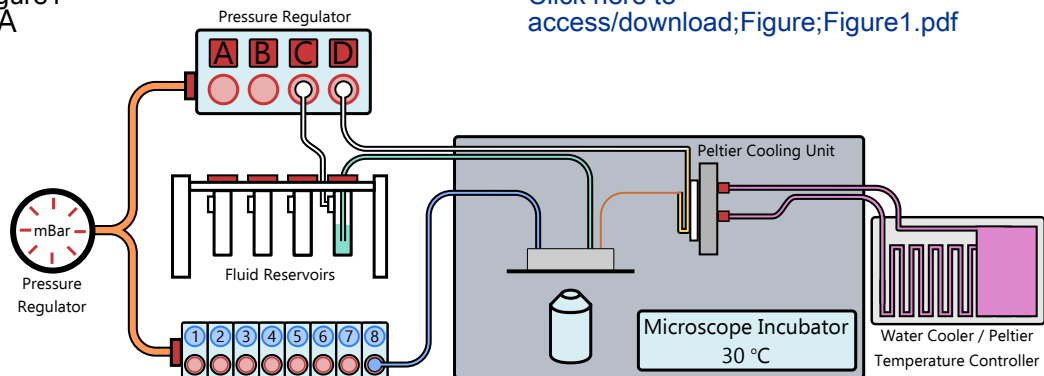920   *Biotechnology* **19,** 751–755 (2001).

921 21. Oberholzer, T., Nierhaus, K. H., Luisi, P. L. Protein expression in liposomes. *Biochemical*
922   *and Biophysical Research Communications* **261,** 238–241 (1999).

923   22.   Yelleswarapu, M. et al. Sigma factor-mediated tuning of bacterial cell-free synthetic
924   genetic oscillators. *ACS Synthetic Biology* **7,** acssynbio.8b00300 (2018).

925   23.   Karig, D. K., Iyer, S., Simpson, M. L., Doktycz, M. J. Expression optimization and synthetic
926   gene networks in cell-free systems. *Nucleic Acids Research* **40,** 3763–3774 (2012).

927   24.   Dunlop, M. J. et al. Distinct timescales of RNA regulators enable the construction of a
928   genetic pulse generator. *Biotechnology and Bioengineering* (2019).
929   doi:10.1002/bit.26918

930   25.   Yoshiyama, T., Ichii, T., Yomo, T., Ichihashi, N. Automated in vitro evolution of a
931   translation-coupled RNA replication system in a droplet flow reactor. *Scientific Reports* **8,**
932   1–8 (2018).

933   26.   Iyer, S., Karig, D. K., Norred, S. E., Simpson, M. L., Doktycz, M. J. Multi-input regulation
934   and logic with T7 promoters in cells and cell-free systems. *PLoS ONE* **8,** 1–12 (2013).

935   27.   de los Santos, E. L. C., Meyerowitz, J. T., Mayo, S. L., Murray, R. M. Engineering
936   Transcriptional Regulator Effector Specificity Using Computational Design and In vitro
937   Rapid Prototyping: Developing a Vanillin Sensor. *ACS Synthetic Biology* **5,** 287–295
938   (2016).

939   28.   Guo, S., Murray, R. M. Construct functional feedforward loop biological circuits in a cell-
940   free system and in cells. *ACS Synthetic Biology* acssynbio.8b00493 (2019).
941   doi:10.1021/acssynbio.8b00493

942   29.   Hughes, R. A., Ellington, A. D. Synthetic DNA synthesis and assembly: putting the
943   synthetic in synthetic biology. *Cold Spring Harbor Perspectives in Biology* **9,** (2017).

944   30.   Garamella, J., Marshall, R., Rustad, M., Noireaux, V. The all E. coli TX-TL toolbox 2.0: A
945   platform for cell-free synthetic biology. *ACS Synthetic Biology* **5,** 344–355 (2016).

946   31.   Takahashi, M. K. et al. Characterizing and prototyping genetic networks with cell-free
947   transcription-translation reactions. *Methods* **86,** 60–72 (2015).

948   32.   Lee, J. W. et al. Creating single-copy genetic circuits. *Molecular Cell* **63,** 329–336 (2016).

949   33.   Gibson, D. G. et al. Complete chemical synthesis, assembly, and cloning of a mycoplasma
950   genitalium genome. *Science* **319,** 1215–1220 (2008).

951   34.   Sun, Z. Z., Yeung, E., Hayes, C. A., Noireaux, V., Murray, R. M. Linear DNA for rapid
952   prototyping of synthetic biological circuits in an Escherichia coli based TX-TL cell-free
953   system. *ACS Synthetic Biology* **3,** 387–397 (2014).

954   35.   Georgi, V. et al. On-chip automation of cell-free protein synthesis: new opportunities due
955   to a novel reaction mode. *Lab on a Chip* **16,** 269–281 (2016).

956   36.   Niederholtmeyer, H., Stepanova, V., Maerkl, S. J. Implementation of cell-free biological
957   networks at steady state. *Proceedings of the National Academy of Sciences of the United*
958   *States of America* **110,** 15985–15990 (2013).

959   37.   A. S. Spirin, V. I. Baranov, L. A. Ryabova, S. Y. Ovodov, and Y. B. A. A continuous cell-free
960   translation system capable of producing polypeptides in high yield. *Science* **242,** 1162–
961   1164 (1988).

962   38.   Karzbrun, E., Tayar, A. M., Noireaux, V., Bar-Ziv, R. H. Programmable on-chip DNA
963   compartments as artificial cells. *Science* **345,** 829–832 (2014).

964   39.   Timm, A. C., Shankles, P. G., Foster, C. M., Doktycz, M. J., Retterer, S. T. Toward
965   microfluidic reactors for cell-free protein synthesis at the point-of-care. *Small* **12,** 810–
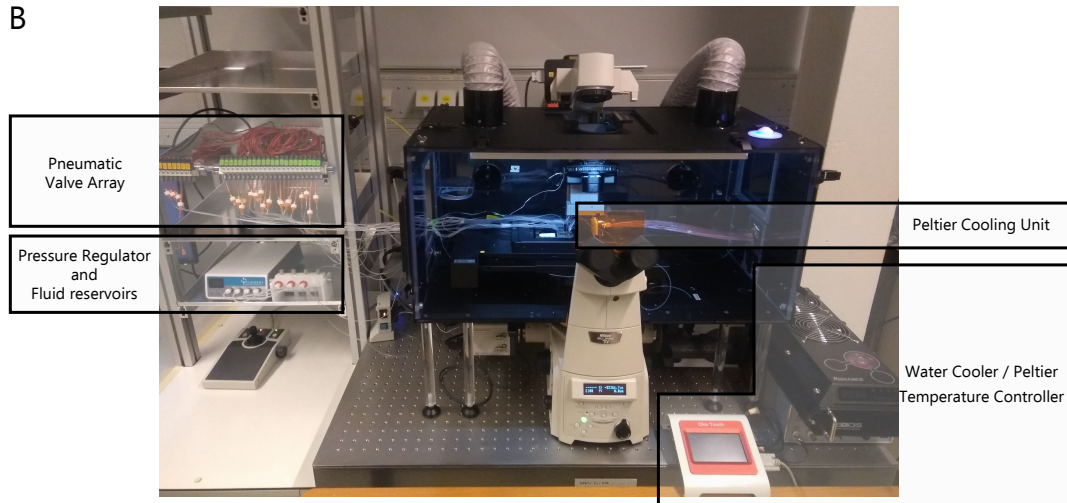966   817 (2016).

967 40. Doktycz, M. J., Foster, C. M., Retterer, S. T., Timm, A. C., Shankles, P. G. Characterization
968   of extended channel bioreactors for continuous-flow protein production. *Journal of*
969   *Vacuum Science & Technology B, Nanotechnology and Microelectronics: Materials,*
970   *Processing, Measurement, and Phenomena* **33,** 06FM02 (2015).
971 41. Khnouf, R., Beebe, D. J., Fan, Z. H. Cell-free protein expression in a microchannel array
972   with passive pumping. *Lab on a chip* **9,** 56–61 (2009).
973 42. Siuti, P., Retterer, S. T., Doktycz, M. J. Continuous protein production in nanoporous,
974   picolitre volume containers. *Lab on a Chip* **11,** 3523–3529 (2011).
975 43. Kaminski, T. S., Scheler, O., Garstecki, P. Droplet microfluidics for microbiology:
976   techniques, applications and challenges. *Lab on a Chip* **16,** 2168–2187 (2016).
977 44. Shin, J., Noireaux, V. An E. coli cell-free expression toolbox: Application to synthetic gene
978   circuits and artificial cells. *ACS Synthetic Biology* **1,** 29–41 (2012).
979 45. Niederholtmeyer, H. et al. Rapid cell-free forward engineering of novel genetic ring
980   oscillators. *eLife* **4,** 1–18 (2015).
981 46. Galas, J., Haghiri-Gosnet, A.-M., Estévez-Torres, A. A nanoliter-scale open chemical
982   reactor. *Lab on a Chip* **13,** 415–423 (2013).
983 47. Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L., Leibler, S. Monolithic Microfabricated
984   Valves and Pumps by Multilayer Soft Lithography. *Science* **288,** 113–116 (2004).
985 48. Brower, K. et al. An open-source, programmable pneumatic setup for operation and
986   automated control of single- and multi-layer microfluidic devices. *HardwareX* **3,** 117–134
987   (2018).
988 49. White, J. A., Streets, A. M. Controller for microfluidic large-scale integration. *HardwareX*
989   **3,** 135–145 (2018).
990

Figure1

A

Pressure Regulator

A B C D

Fluid Reservoirs

Pressure Regulator

mBar

1 2 3 4 5 6 7 8

Peltier Cooling Unit

Microscope Incubator
30 ℃

Water Cooler / Peltier
Temperature Controller

B

Pneumatic
Valve Array

Pressure Regulator
and
Fluid reservoirs

Peltier Cooling Unit

Water Cooler / Peltier
Temperature Controller

Figure2

Click here to access/download;Figure;Figure2.pdf ⬇

Controller Connector Pins

1/4" Stopper

1 2 3 4 5 6 7 8

Compressed Air Supply Port (1/4" Push Connection)

Polyurethane Tubing (OD:4mm,ID:2.5mm)

3/32" Barb to Male Luer Connector

Luer Stub (23 ga, 1/2")

Tubing (OD:0.06", ID:0.02")

Figure3

Figure4

Figure 4

Figure5

Control Layer (45 μm)   Flow Layer (25 μm)

Figure 6

Figure8

Figure9

Fluorescence Intensity of deGFP Expression
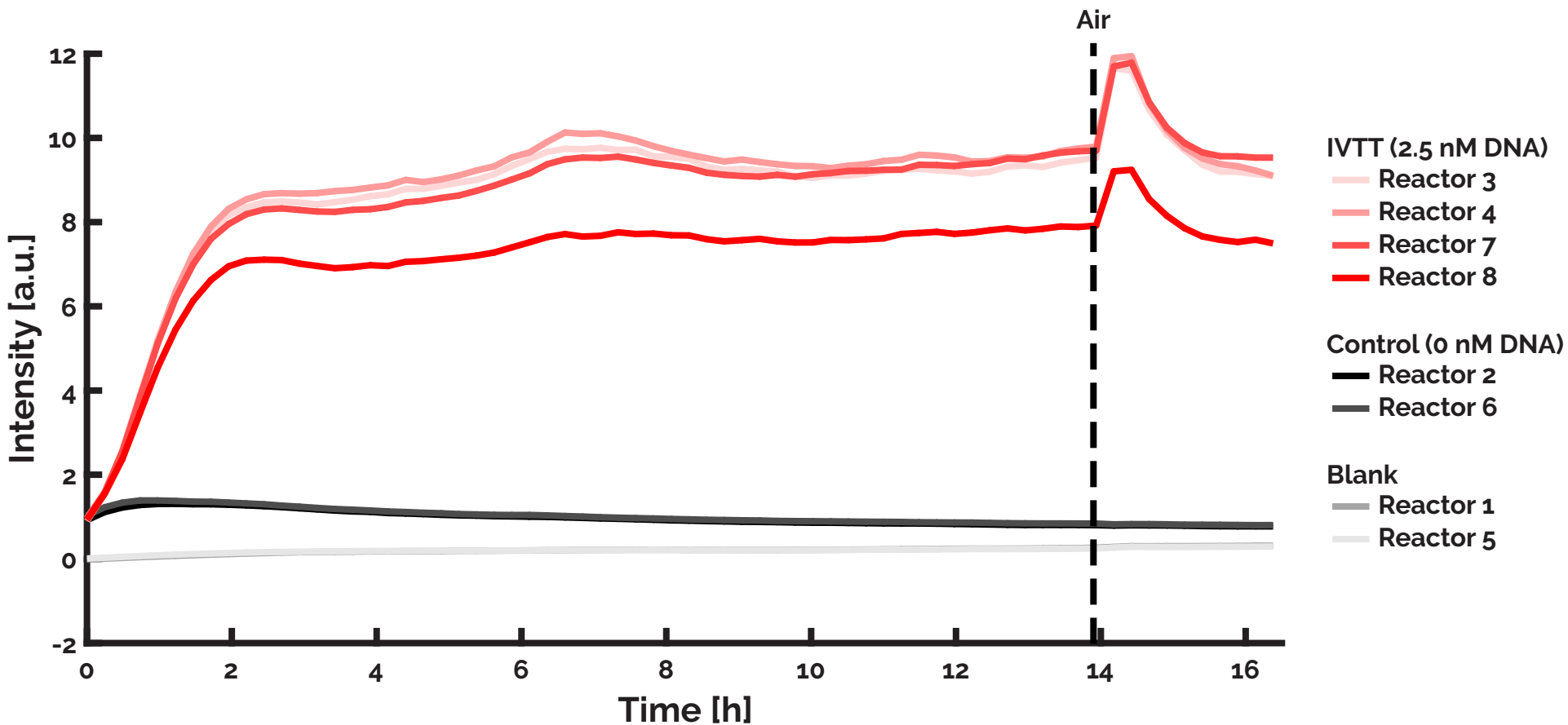
Name of Material/ Equipment	Company	Catalog Number
Reagents		
Acetone	VWR	20063.365
AZ 40 XT	Merck KGaA (Darmstadt, Germany)	-
AZ 726 MIF Developer	Merck KGaA (Darmstadt, Germany)	-
Isopropanol	Merck KGaA (Darmstadt, Germany)	109634
Microscope slides	VWR	ECN 631-1550
mr Dev 600	Microresist Technology GmbH (Berlin, Germany)	-
Silicon Free Heat Sink Grease	Circuit Works	CW7270
Silicon wafers	Silicon Materials	-
SU-8 3050	Microchem Corp. (Newton, MA)	-
Sylgard 184 Elastomer Kit (PDMS)	The Dow Chemical Company	01317318
trichloro(1H,1H,2H,2H-perfluorooctyl)silane	Sigma-Aldrich	448931-10G
Equipment		
4 channel digital input/output module	WAGO Kontakttechnik GmbH	750-504
Camera lens	The Imaging Source	-
Compression fitting	Koolance, Inc.	FIT-V06X10
Controller end module	WAGO Kontakttechnik GmbH	750-600
Device connecting tubing	Saint-Gobain Performance Plastics	AAD04103
Device connector pins	Unimed SA (Lausanne, Switzerland)	200.010-A

Item	Manufacturer	Catalog Number
Ethernet Controller	WAGO Kontakttechnik GmbH	750-881
Female bus connector	Encitech	DTCK15-DBS-K
Fluid reservoirs	Fluigent	Fluiwell-4C
Fluigent pressure system	Fluigent	MFCS-EZ
Hg short arc lamp	Advanced Radiation Corporation	-
Hot plate	Torrey Pines Scientific	HP61
Inverted microscope	Nikon Instruments	Eclipse Ti-E
LabVIEW Software	de Greef Lab, Eindhoven University of Technology	https://github.com/tfadgreef/Microfluidic-Device-Control-Software
Liquid coolant	Koolance, Inc.	LIQ-705CL-B
Luer stubs	Instech Laboratories, Inc.	LS23
Male Luer to barb connectors	Cole Parmer	45505-32
Matlab Software	de Greef Lab, Eindhoven University of Technology	https://github.com/tfadgreef/Microfluidic-Device-Control-Software
Microcamera	The Imaging Source	DMK 42AUC03
Microscope camera	Hamamatsu Photonics	OrcaFlash4.0 V2 (C11440-22CU)
Orbital shaker	Cole Parmer	EW-513000-05
Oven	Thermo Scientific	Heraeus T6P 50045757
Oxygen plasma asher	Quorum Technologies	K1050X
PDMS puncher	SYNEO	Accu-Pucnh MP10
PEEK tubing	Trajan	1301005001-5F
Peltier element	European Thermodynamics	APH-127-10-25-S
Peltier temperature controller	Warner Instruments	CL-100
Photomask	CAD/Art Services, Inc.	-
Photomask Design	Maerkl Lab, EPFL	https://zenodo.org/record/886937#.XBzpA8-2nOQ
Pneumatic valve array	FESTO	-
Power adapter	Koolance, Inc.	ADT-EX004S
PTFE tubing	Cole Parmer	06417-21
Punching pin	SYNEO	CR0320245N21R4
PVC Tubing	Koolance, Inc.	HOS-06CL
Single edge blades	GEM Scientific	-

Soft tubing	Fluigent	-
Spin coater	Laurell Technologies Corporation	WS-650MZ-23NPPB
Stereo microscope	Olympus Corporation	SZ61
Thermistor cable	Warner Instruments	TA-29
UV exposure system	ABM, USA	-
Vacuum pump	Vacuumbrand GmbH	MD1C
Water cooled cold plate block	Koolance, Inc.	PLT-UN40F
Water cooler	Koolance, Inc.	EX2-755
Weighing scales	Sartorius	M-prove

Comments/Description

Positive Photoresist

Developer Positive Photoresist

Developer Negative Photoresist
Thermal Compound
<1-0-0> orientation, 100 mm diameter, 525 µm thickness

Negative Photoresist

8x

Fitting for tubing with 6mm ID and 10mm OD. 4x

0.02" ID, 0.06" OD, Tygon Tubing (ND-100-80)

AISI 304 tubing, 0.35mm ID, 0.65mm OD, 8mm L

15 pole female bus connector

0 - 345 mbar

350W

3/32" ID

0.005" ID, 1/32" OD, Red

1x 22 valve array and 1x 8 valve array, Normally closed valves.
110/220V AC Power Adapter
#24 AWG Thin Wall PTFE
OD: 0.032" (0.8128 mm), ID: 0.024" (0.6090 mm)
6 mm ID, 10 mm OD

Supplied with fluid reservoirs. (1 mm ID, 3mm OD)

Cable with bead thermistor
Near UV Exposure System, 350W

jove

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

JOURNAL OF
VISUALIZED EXPERIMENTS

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	A Multilayer Microfluidic Platform for the Conduction of Prolonged Cell-Free Gene Expression
Author(s):	Ardjan J van der Linden, Maaruthy Yelleswarapu, Pascal A Pieters, Zoe Swank, Wilhelm T S Huck, Sebastian J Maerkl, Tom F A de Greef

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access   ☒ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1.   **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2.   **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3.   **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

612542.6   For questions, please contact us at submissions@jove.com or +1.617.945.9051.

jove
JOURNAL OF
VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4.   Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5.   Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6.   Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7.   Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8.   Protection of the Work. The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9.   Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10.   Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11.   JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove
JOURNAL OF
VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12.   **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to

the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13.   **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14.   **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name: *Tom J. A. de Greef*

Department: *Biomedical Engineering*

Institution: *Institute for complex Molecular Systems*

Title: *Prof.*

Signature: [signature]   Date: *20/12/2018*

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1.   Upload an electronic version on the JoVE submission site
2.   Fax the document to +1.866.381.2236
3.   Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial Comments:

We would like to thank the editor for the comments provided and will answer his concerns below:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have taken the time re-read the manuscript and have removed any spelling / grammatical mistakes we encountered.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
We have contacted the editors of both journals from which we have adapted figures. Both editors have provided us with explicit permission to use the figures. Proof of these permissions have been uploaded as separate files.

3. JoVE cannot publish manuscripts containing commercial language. This includes company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include AZ, WAGO, milliQ, Nikon, etc.
We have taken care to remove any mentions to specific commercial products.

4. Please add a one-line space between each of your protocol steps.
We have included spacing between each of the protocol steps conform to the

5. Step 4.1.1: Please ensure that all text is written in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We have rewritten this step to ensure that it comprises solely of the imperative tense.

6. 4.1.2: Please ensure that all text is written in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We have rewritten this step to ensure that it comprises solely of the imperative tense.

7. 4.2.4: Please ensure that all text is written in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We have rewritten this step to ensure that it comprises solely of the imperative tense.

8. 4.2.5: Please ensure that all text is written in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We have rewritten this step to ensure that it comprises solely of the imperative tense.

9. 4.3.1: Please ensure that all text is written in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We have rewritten this step to ensure that it comprises solely of the imperative tense.

10. 5.2.1: Please ensure that all text is written in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We have rewritten this step to ensure that it comprises solely of the imperative tense.

11. 6.3.1: Please ensure that all text is written in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We have rewritten this step to ensure that it comprises solely of the imperative tense.

12. 6.3.3: Please ensure that all text is written in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We have rewritten this step to ensure that it comprises solely of the imperative tense.

13. Why are there two steps 6.3.3?
We have restructured this section and have removed the incorrect numbering here.

14. 6.4.1-6.4.3: Please ensure that all text is written in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We have rewritten this step to ensure that it comprises solely of the imperative tense.

15. Please expand step 7 into more sub-steps to include enough details.
We have expanded step 7 into multiple sub-steps, using the imperative sense to guide readers through this process.

16. Please do not abbreviate journal titles for filming.
We shall take care to avoid the use of (abbreviated) journal titles during filming.

17. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
We have selected the most cohesive 2.75 pages of the protocols which highlight the most challenging and critical elements of our method.

Reviewer #1:
We thank the reviewer for their positive comments regarding our manuscript and are especially pleased to hear that the reviewer sees our work as a valuable resource for other researchers in the field. A response to the reviewers question has been provided below.

Are there CAD files available for the microfluidic design?
The microfluidic device designs are available online and a link has been provided within the materials list supplied with the manuscript. An explicit reference to the device designs is included in protocols sections 1 and 2. In addition, a schematic of the microfluidic device design is provided in figure 5 to provide readers with an overview of the device architecture whilst reading the manuscript.

Reviewer #2:
We thank the reviewer for their positive comments, and have reviewed the manuscript once more to remove any grammatical errors. Specifically, we have corrected the issues highlighted by the reviewer as detailed below.

1) Line 284: "Using a scapel to cut out"
We agree that this sentence does not read easily and have amended it to read as follows: "Using a scalpel, remove each of the four devices from the PDMS on the flow layer wafer."

2) Line 317: "atop"
We have adapted the sentence to read as follows: "Weights can be placed on top of the devices to improve adhesion of the device"

3) Line 448: A comma is missing
We have adapted the sentence to read as follows: "When pressure is applied to the reservoirs via the pressure regulator software, the reagents will be forced into the microfluidic device."

4) Line 583: This sentence is confusing, GamS was introduced individually?
We understand that the sentence may be confusing to readers due to the lack of background information provided about the GamS addition. When working with linear DNA templates, the addition of GamS to IVTT reaction solutions is critical in limiting the degradation of the DNA templates by exonucleases. Since GamS is not present in the E.coli based IVTT solutions we use, it needs to be added to the reaction solution separately prior to beginning the experiments. It is not necessary to add the GamS when working with plasmids. The sentence referring to the GamS has been modified as follows in the hope that any ambiguity in the protocol has been removed.
"The IVTT reaction mixture accommodates all the components required to successfully express proteins however, purified GamS was added to the reaction mixture – at a final concentration of 1.3 μM - prior to the loading thereof into the microfluidic device. The addition of the GamS protein serves to minimise the degradation of linear DNA species when performing the experiments."

Reviewer #3:
We thank the reviewer for their positive comments regarding the work presented here and take this opportunity to thank them for their helpful feedback. We agree with the concerns presented by the reviewer and have outlined our responses to these concerns below.

1) In the introduction, citing more researchers who have constructed cell-free gene circuits may help to broaden appeal. A few come to mind beyond the few that are already included: Julius Lucks, David Karig, Tetsuya Yomo, Sukanya Iyer.
We thank the review for this comment and agree that more references could be added to broaden the appeal of the manuscript. As such we have added the following references to the paper on line 89 following this sentence: "In this manner, a biologically relevant, albeit artificial, environment is created within which synthetic networks can be tested".

1.   Yelleswarapu, M. *et al.* Sigma factor-mediated tuning of bacterial cell-free synthetic genetic oscillators. *ACS Synth. Biol.* **7,** acssynbio.8b00300 (2018).
2.   Karig, D. K., Iyer, S., Simpson, M. L. & Doktycz, M. J. Expression optimization and synthetic gene networks in cell-free systems. *Nucleic Acids Res.* **40,** 3763–3774 (2012).
3.   Dunlop, M. J. *et al.* Distinct timescales of RNA regulators enable the construction of a genetic pulse generator. *Biotechnol. Bioeng.* (2019). doi:10.1002/bit.26918
4.   Yoshiyama, T., Ichii, T., Yomo, T. & Ichihashi, N. Automated in vitro evolution of a translation-coupled RNA replication system in a droplet flow reactor. *Sci. Rep.* **8,** 1–8 (2018).

5. Iyer, S., Karig, D. K., Norred, S. E., Simpson, M. L. & Doktycz, M. J. Multi-Input Regulation and Logic with T7 Promoters in Cells and Cell-Free Systems. *PLoS One* **8,** 1–12 (2013).
6. De Los Santos, E. L. C., Meyerowitz, J. T., Mayo, S. L. & Murray, R. M. Engineering Transcriptional Regulator Effector Specificity Using Computational Design and in Vitro Rapid Prototyping: Developing a Vanillin Sensor. *ACS Synth. Biol.* **5,** 287–295 (2016).

2) Around line 109, a bit more expansion on this method in the context of other approaches would be helpful. Also, how does this microfluidic approach compare to other microfluidic approaches, e.g. Timm et al, Small 2015 (https://doi.org/10.1002/smll.201502764)?

We thank the reviewer for their comment, and have added two references regarding the use of continuous flow microfluidics to this sentence: "The versatility of IVTT systems enables multiple alternative methods by which prolonged IVTT reactions can be performed, ranging from continuous flow to droplet based methods as well as simpler dialysis approaches[2,29,36–39]." Furthermore, we elaborated on the differences between previously reported methods and that which we report here by adding the following sentences:

"The versatility of IVTT systems enables multiple alternative methods by which prolonged IVTT reactions can be performed, ranging from continuous flow to droplet based methods as well as simpler dialysis approaches[2,29,36–39]. The application of microfluidic devices offers users increased control over their reactions whilst increasing the throughput and minimizing costs[34,40,41], with each specific approach having its own advantages. The use of continuous flow can be easily optimized for increasing expression yields however, the inability to effectively remove specific reaction products makes the study of dynamic behaviour non-trivial[38]. Whilst employing droplet based microfluidic systems allows high-throughput screening of novel networks, the difficulty of supplying fresh reagents to the reaction result in droplets resembling small volume batch reactions42. Dialysis reactors allow the introduction of fresh reagents as well as the removal of some reaction products however, RNA molecules and larger proteins accumulate within the reactor, being too large to diffuse through the membrane pores. Furthermore, large volumes of reagents are required to sustain these reactions for prolonged periods[29,43]."

3) I understand what the authors are getting at with the mention of negative feedback, but I would not refer to 'feedback'. Rather, I would suggest referring to negative regulation, ON-OFF transitions, or fluctuating dynamics. Negative feedback systems may have monotonic dynamics, and negative feedback was also implemented in batch (https://doi.org/10.1093/nar/gkr1191).

We thank the review for this critical comment and to avoid any misunderstandings we have replaced the mention of 'negative feedback' with the reviewers suggestion of 'negative regulation'.

4) Please specify the size and type of silicon wafer used.

1.1 *We have included the specifications of the silicon wafer used in both the protocols section as well as in the materials list provided along with the main manuscript. The reference to the silicon wafer now reads: "Place a clean silicon wafer (100 mm diameter, <1-0-0> oriented, 525 µm thickness) in a preheated oven set to 250 °C and leave the wafer to dehydrate overnight (~16 h)."*

5) Please clarify "thermal paste" in the Figure 4 caption.

We have clarified the type of thermal paste used in the figure caption, as well as adding the specific thermal paste to the materials list. The caption now reads: "To remove excess heat produced by the Peltier element, the hot-face of the Peltier is placed against a water cooled block, with the addition of silicone free heat sink grease thermal paste ensuring optimal heat transfer between the two faces."

We would like to thank the editor for the comments provided and will answer his concerns below:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have taken the time to re-read the manuscript and have removed any spelling / grammatical mistakes we encountered.

2. There is a 10 page limit for the Protocol. Please shorten the protocol to meet the limit.
We have put considerable effort into ensuring that the protocols no longer exceed 10 pages in length. We have done so while including the specified 1-line spacing between all protocol steps. (We have checked this when accepting all reviews and comments, and found that the text is indeed less than 10 pages in length, however the occasional double spacing may lead to an increase in the length. We have attempted to remove all double spaces, however this is not always clear in the review tracking process.)

3. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
We have selected the parts of our protocol which we feel are most important for readers of the manuscript, and have checked the length of the highlighted sections. Including the spacing, the length of the selected steps is 2.5 pages.

4. Please split some long steps into two or more steps so that each step contains 2-3 actions and is less than 4 line.
During the rewriting of the protocols sections, many of the longer steps have been reduced in length, with "non-critical" being removed. While we have made every attempt to ensure that the length of each individual step does not exceed 4 lines, this was not always possible due to the complexity of the procedure at hand.

5. Please come some short steps so that each step contains 2-3 actions.
During the restructuring of the protocols section we have also combined many shorter / simpler steps into a single step to aid in shortening the protocols section.

6. Please do not abbreviate journal titles for all references.
We have removed any journal abbreviations from the references, and are now providing the full journal title as per your request.

7. Step 6.2.2: Please write this step in the imperative tense.
During the restructuring this step has been combined with another step, and care has been taken to write the step in the imperative tense.

8. 6.4.3: Please write this step in the imperative tense.
This step has been combined with another step such that the readability of the protocols is improved. The new step has been written in the imperative tense.

9. 7.4: Please write this step in the imperative tense.
This step has been removed and the critical information has been restructured into a note following another step in the protocol.

10. Figure 6 was never mentioned in the manuscript.
We thank the editor for this astute observation and have rectified this error by adding a reference to the figure in the appropriate part of the manuscript.

From:	PNAS Permissions
To:	Linden, A.J. van der
Subject:	RE: Permission Request
Date:	woensdag 13 februari 2019 21:06:09

Thank you for your message. Permission is granted for your use of the material as described in your request. Please include a full citation for the original PNAS article when reusing the material. Because this material published after 2008, a copyright note is not needed. There is no charge for this material, either. Let us know if you have any questions.

Sincerely,

Kay McLaughlin for
Diane Sullenberger
PNAS Executive Editor

From: Linden, A.J. van der <A.J.v.d.Linden@tue.nl>
Sent: Wednesday, February 13, 2019 5:01 AM
To: PNAS Permissions <PNASPermissions@nas.edu>
Subject: Permission Request

Dear Sir / Madam,

We would like to request your permission to publish an adaptation of Figure S8, from the following article

https://www.pnas.org/content/110/40/15985

which has been previously published in PNAS, for open-access publication in The Journal of Visualized Experiments (JoVE).

With thanks in advance,

Ardjan van der Linden

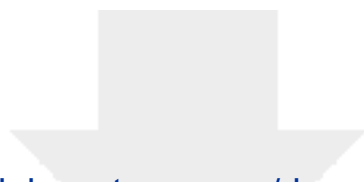|	
From:	support@services.acs.org
To:	Linden, A.J. van der
Subject:	RE: Figure Usage Request
Date:	dinsdag 12 februari 2019 12:45:26

Dear Dr. Ardjan van der Linden,

Thank you for contacting ACS Publications Support.

Your permission requested is granted and there is no fee for this reuse.
In your planned reuse, you must cite the ACS article as the source, add this direct link
<https://pubs.acs.org/doi/10.1021/acssynbio.8b00300 >, and include a notice to readers that
further permissions related to the material excerpted should be directed to the ACS.

Please do not hesitate to contact us if you need any further assistance.

Sincerely,

Ivan Ostojic
ACS Publications
Customer Services & Information
Website: https://help.acs.org

-----Original Message-----
Priority: None
From: (Linden, A.J. van der) A.J.v.d.Linden@tue.nl
Sent: 2/12/2019
To: support@services.acs.org
Cc:
Subject: Figure Usage Request
Dear Sir/Madam,

We would like to request your permission to publish an adaptation of Figure S20, from the
following article

https://pubs.acs.org/doi/10.1021/acssynbio.8b00300

which has been published under the ACS Author Choice license for open-access publication in
The Journal of Visualized Experiments (JoVE).

With thanks in advance,

Ardjan van der Linden

{CMI: MCID628151}

Labview Code

Click here to access/download
Supplemental Coding Files
ConvertDesiredRefreshRatio.vi

Labview Code

Labview Code
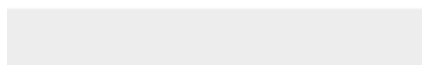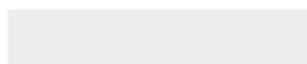
Click here to access/download
Supplemental Coding Files
CurrentSwitchValues_GlobalVariable.vi

Labview Code

Click here to access/download
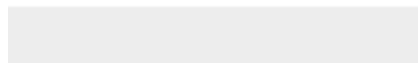**Supplemental Coding Files**
DeterminePingCommand.vi

Labview Code

Click here to access/download
Supplemental Coding Files
DetermineTimeSinceLastExecution.vi

Labview Code

Click here to access/download
Supplemental Coding Files
dummy_sender.exe

Labview Code

Click here to access/download
Supplemental Coding Files
dummy_stopper.exe

Labview Code

Click here to access/download
Supplemental Coding Files
MainInterface.vi

Labview Code

Click here to access/download

Supplemental Coding Files

SequenceSelector_Flush.vi

Labview Code

Labview Code
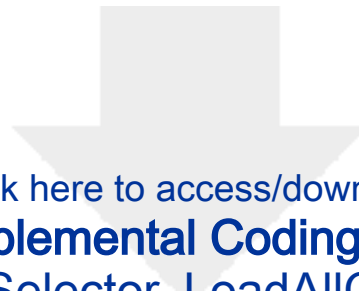
Click here to access/download
Supplemental Coding Files
SequenceSelector_LoadAllChambers.vi

Labview Code
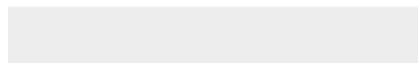
Click here to access/download

Supplemental Coding Files

SequenceSelector_LoadPerReactor.vi

Labview Code
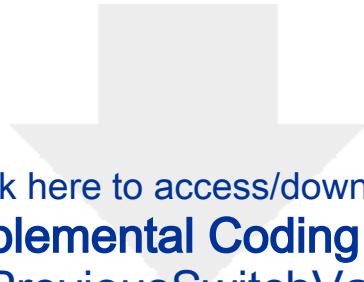
Click here to access/download
Supplemental Coding Files
StorePreviousSwitchValues.vi

Labview Code

Click here to access/download
Supplemental Coding Files
TCPRead_Write.vi

Labview Code

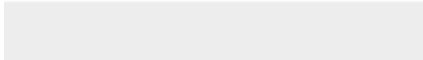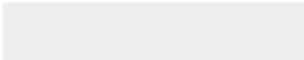Click here to access/download
Supplemental Coding Files
TCPRead_Write_GlobalVariables.vi

Matlab Analysis/Calibration Script

Click here to access/download
Supplemental Coding Files
calibrationScript.m

Matlab Analysis/Calibration Script

Click here to access/download
Supplemental Coding Files
intensityDetermination.m

Matlab Analysis/Calibration Script

Click here to access/download
Supplemental Coding Files
myGUI.m

Matlab Analysis/Calibration Script

Matlab Analysis/Calibration Script

Click here to access/download
Supplemental Coding Files
regionOfInterestDetermination.m

Matlab Analysis/Calibration Script