

# Engineering a living biomaterial via bacterial surface capture of environmental molecules

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## Abstract

Synthetic biology holds significant potential in biomaterials science as synthetically engineered cells can produce new biomaterials, or alternately, can function as living components of new biomaterials. Here, we describe the creation of a new biomaterial that incorporates living bacterial constituents that interact with their environment using engineered surface display. We first developed a gene construct that enabled simultaneous expression of cytosolic mCherry and a surface-displayed, catalytically active enzyme capable of covalently bonding with benzylguanine (BG) groups. We then created a functional living material within a microfluidic channel using these genetically engineered cells. The material forms when engineered cells covalently bond to ambient BG-modified molecules upon induction. Given the wide range of materials amenable to functionalization with BG-groups, our system provides a proof-of-concept for the sequestration and assembly of BG-functionalized molecules on a fluid-swept, living biomaterial surface.

**Key words:** surface display; interfaces; microfluidics; biomaterials.

## 1. Introduction

Advances in biological engineering and materials science have enabled the creation of new biomaterials capable of integrating with living systems and augmenting their behavior.<sup>1</sup> In contrast to biocompatible materials, which have been utilized for millennia,<sup>2</sup> modern biomaterials are engineered with enhanced functionality. The materials are able to selectively augment living microenvironments such as the tumor ecosystem<sup>3</sup> and local environment surrounding neural implants.<sup>4</sup> With a deepening understanding of cellular biology and material interactions,<sup>5</sup> biocompatible materials such as polyethylene and bio-inert alloys like titanium<sup>6,7</sup> have given way to more nuanced bioactive materials such as microbial resistant hydroxyapatite<sup>8</sup> and degradable conductive polymers.<sup>9,10</sup> Taken as a whole,

biomaterials show great promise in not only medicine,<sup>11–13</sup> but also bioprocessing and drug development.<sup>14</sup>

Simultaneously, the field of synthetic biology has given scientists and engineers the ability to selectively control the behavior of living cells. From the foundational genetic toggle switch<sup>15</sup> and repressilator<sup>16</sup> to more intricate circuit topologies such as predator–prey systems,<sup>17–21</sup> the field has developed to include a broad library of genetic circuits and constructs. In the process, synthetic biology has yielded insight into fundamental biological principles<sup>22</sup> while enabling unprecedented bioprocessing<sup>23</sup> and disease diagnostic<sup>24</sup> advances.

Furthermore, synthetic biology has allowed scientists and engineers to begin to develop new biomaterials, designed at both a material and genetic level.<sup>25,26</sup> These biomaterials show

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great promise, combining the advantages of self-replicating life with genetically engineered behavior. Deliberate biofilm dispersion<sup>27</sup> and nanoscale material fabrication<sup>28,29</sup> are just a few of the exciting new directions for synthetic biology in the creation of self-healing, adaptive or responsive living materials. Here, we report the development of a biomaterial surface coating consisting of living cells capable of sequestering chemicals from their environment. This material is based on a synthetic system for outputting intracellular genetic events through the surface-display of enzymes.

The cellular envelope of *Escherichia coli* is made up of two phospholipid bilayers—the outer surface membrane and cytoplasmic membrane—separated by the periplasmic space.<sup>30</sup> With this structure in mind, we designed and built synthetic components consisting of chimeric fusion proteins that could embed in the cellular envelope and reliably display a functionally active enzyme on the *E. coli* surface. Although there are many protein structures that span both membranes, we leveraged an existing engineered surface-display anchor that presents protein structures extracellularly, anchors within the cellular envelope and does not interact with the inner plasma membrane.<sup>30</sup> This chimeric fusion protein, lpp-ompA, enables C-terminal fusion and display of large proteins.<sup>31</sup> It was first successfully demonstrated to express  $\beta$ -lactamase on the surface of *E. coli*,<sup>32</sup> and consists of the first nine N-terminal amino acids of a lipoprotein (lpp) fused to amino acids 46–159 of outer membrane protein A (ompA). We created a new fusion protein based on lpp-ompA, allowing us to express modified human O<sup>6</sup>-alkylguanine DNA alkyltransferase (hAGT), also known as the SNAP-tag, on the surface of Gram-negative bacteria.

SNAP is a useful tool in tagging functional proteins in living cells, with capabilities that complement other protein tags such as the polyhistidine tag, GFP or other fluorescent proteins.<sup>33</sup> SNAP forms a covalent bond with benzylguanine (BG) groups in its environment. The SNAP-tag was derived from O<sup>6</sup>-alkylguanine-DNA-alkyltransferase (AGT), a human DNA repair enzyme.<sup>34</sup> AGT's binding affinity for BG was initially demonstrated in 2003 by Keppler et al.<sup>34</sup> and was later improved in 2006 by Gronemeyer et al.,<sup>35</sup> resulting in an optimized protein (SNAP) with a 50-fold increase in affinity for BG. The SNAP-tag has since been widely utilized in a variety of studies ranging from medical applications, such as tumor targeting<sup>36,37</sup> and drug delivery,<sup>38</sup> to basic scientific research on protein network and interactions.<sup>39,40</sup> In 2010, *E. coli* cells expressing SNAP in their cytosol demonstrated successful live labelling with BG-MR121 fluorescent dyes.<sup>41</sup> Beyond this previous study, there has been minimal use of the SNAP-tag technology in bacteria.

By fusing SNAP with the lpp-ompA protein, we engineered *E. coli* with a surface-displayed, BG-binding enzyme. The genetic construct endowed cells with a phenotypic expression platform spatially discrete in comparison to traditional cytosolic fluorescent proteins. Additionally, the construct conferred an inducible ability that enabled cells to be selectively labelled by BG-modified fluorescent chemical dyes while simultaneously upregulating intracellular fluorescent protein (i.e. mCherry) expression. As BG-modified dyes cannot be transported into the bacterial cytosol due to size constraints, the results described in this work confirmed that the SNAP enzyme was transported to the cell's surface, while providing a new method for selectively discretizing surface and cytosolic genetic outputs in bacteria. In other synthetic biology studies, genetic circuit outputs have been coupled to regulatory gene circuits to probe how regulation dynamics and cellular behavior change over time.<sup>15,16</sup> Here, we focused on utilizing the spatial heterogeneity of the cell

(i.e. the spatial domains associated with the cytosol and the outer membrane) to engineer synthetic gene constructs to deliberately form a biomaterial.

## 2. Materials and methods

### 2.1 Media, reagents, growth and induction conditions

All *E. coli* cells were grown in Luria-Bertani (LB) Broth, Miller (Fisher Scientific). Antibiotic was added after cooling to 55°C with a final concentration of 50  $\mu$ g/mL, if required. Induction was performed by inoculating new LB media containing the required amounts of arabinose with 1:100 dilution of overnight culture. The samples were then incubated at 37°C for 6 h with agitation.

### 2.2 Bacterial strains and plasmids

Three strains of *E. coli* were used (Supplementary Table S1). *Escherichia coli* NEB Turbo (F' *proA*<sup>+</sup>*B*<sup>+</sup> *lacI*<sup>q</sup>  $\Delta$ lacZM15/*fhuA2*  $\Delta$ (*lac-proAB*) *glnV* *galK16* *galE15* *R*(*zgb-210::Tn10*) *Tet*<sup>S</sup> *endA1* *thi-1*  $\Delta$ (*hdsS-mcrB*)5) (New England Biolabs, Inc.) was used for recombinant plasmid transformation and screening due to the strain's high growth rate. Further gene expression testing was performed in *E. coli* strains MG1655  $\Delta$ lacI  $\Delta$ araBAD, allowing all arabinose to be utilized for induction rather than cell growth and proliferation.

Molecular cloning was performed using components from a system for high speed cloning by Litcofsky et al.<sup>42</sup> The plasmid pKE1-MCS was used as the backbone vector for construction of all subsequent plasmids. Promoter P<sub>BAD</sub> was amplified through polymerase chain reaction (PCR) from plasmid pKLi034. The SNAP gene was amplified from pSNAP-tag<sup>®</sup> (T7)-2 (New England Biolabs, Inc.). A cytosolic expression cassette containing the P<sub>LtetO-1</sub> promoter driving mCherry expression was isolated by restriction digestion from plasmid pWR011 (Supplementary Table S1), and the promoter was subsequently replaced with P<sub>BAD</sub> for inducible cytosolic mCherry expression.

The final plasmids used for testing were pFYS018 and pFYS028, which have P<sub>BAD</sub> driving surface-displayed SNAP and P<sub>BAD</sub> driving surface-displayed SNAP and cytosolic mCherry, respectively.

### 2.3 Cloning and PCR

The methods used to construct the final SNAP surface-display plasmid were based on a 'plug-and-play' methodology developed by Litcofsky et al.<sup>42</sup> Plasmid DNA was extracted using Epoch<sup>®</sup> Biolabs Nucleic Acid Kits. Test cuts with restriction enzymes and Sanger sequencing were performed to verify constructs. Plasmid maps can be found in Supplementary Figures S1–S3).

Touchdown PCR program (Supplementary Table S2) was used for all gene amplification. A 5 min incubation at 95°C followed by 10 min at –80°C were added in front of the touchdown PCR program for whole-cell PCR. Primers can be found in Supplementary Table S3.

### 2.4 Fluorescence labelling

SNAP-Cell<sup>®</sup> 505-Star is a fluorescent label purchased from New England Biolabs, Inc., with an excitation peak at 504 nm and an emission peak at 532 nm. SNAP-Cell<sup>®</sup> 505-Star was an ideal candidate for tests because the label excitation and emission spectra did not result in significant cross-talk with mCherry and the

label binding was restricted to the surface of the cells, eliminating any chance of labeling cytosolic proteins. The following procedures were all based on New England Biolabs, Inc.'s Cellular Labelling (S9103) protocol.

Fifty microliter of DMSO was added to 50 nmol of fluorescent label, resulting in a 1 mM stock solution. The solution was vortexed for 10 min before storing in the dark at  $-20^{\circ}\text{C}$ . Immediately before testing, the frozen stock was diluted 1:200, yielding a labelling medium of 5  $\mu\text{M}$  dye label. The labelling medium was homogenized to reduce background by gentle, repetitive pipetting (10 times in the same vessel). Two hundred microliter of cultured cells were resuspended in 30  $\mu\text{L}$  of labelling medium by centrifuging cell cultures, removing the supernatant and resuspending the cells by repetitive pipetting. The tubes were then incubated at  $37^{\circ}\text{C}$  for 30 min. The cells were washed three times by centrifuging, decanting and resuspending in  $1\times$  Phosphate Buffered Solution (PBS). After the last wash, cells were resuspended in 30  $\mu\text{L}$  of 4% paraformaldehyde for 30 min for fixing. Finally, the reactions were centrifuged, and the supernatant was replaced a final time with PBS for further analysis in the flow cytometer.

## 2.5 Fluorescence detection and imaging

A Becton Dickinson Accuri<sup>TM</sup> C6 outfitted with a 488 nm laser and a 552 nm laser were used for all flow cytometry analysis. Further data processing was performed using FlowJo<sup>TM</sup> software. A Nikon Eclipse Ti-E inverted epifluorescent microscope, with an Andor<sup>®</sup> Zyla scientific CMOS (sCMOS) camera mounted for image acquisition, was used for fluorescence imaging and Nikon's NIS-Elements software was used for capturing and analyzing images.

## 2.6 Microfluidic device fabrication

A poly(dimethylsiloxane) (PDMS) Sylgard<sup>®</sup> 184 elastomer kit was purchased from Ellsworth Adhesives. The pre-polymer and curing agent were well-mixed at a 10:1 ratio. The mixture was degassed in a vacuum chamber for 1 h and poured on a clean mold with the desired channel, patterned with SU-8 photoresist. The mold was then degassed for an additional 1 h before being placed in an  $80^{\circ}\text{C}$  oven for 30 min. The PDMS was carefully removed from the mold, and excess PDMS was removed using a razor blade. The channel was cleaned with Sparkleen (Fisherbrand), rinsed with deionized water, sprayed with 70% ethanol and rinsed again with deionized water. The PDMS was dried with filtered, in-house air before covering with scotch tape until ready for plasma cleaning. Cover slips were cleaned and dried in a similar manner before sealing the microdevices. To seal microdevices, the microchannel and cover slip were plasma cleaned for 1 min, sealed and placed in the oven for 30 min. The resulting device channel dimensions were 100  $\mu\text{m}$  in height with a length and width of 4 mm and 2 mm, respectively.

## 2.7 Microchannel silanization and bio-functionalization

Immediately following sealing of the device, a 10% (3-aminopropyl)triethoxysilane (APTES) in ethanol solution was injected into the channel and incubated at room temperature for 30 min. Channels were then rinsed with 95% ethanol followed by filtered deionized water to remove unreacted APTES. A 2% glutaraldehyde (GA) solution was then added to the channel for 30 min at room temperature before rinsing with filtered PBS. Cells grown overnight were concentrated and resuspended in filtered PBS. The cells were then flowed into the channel and incubated at room temperature for 1 h for attachment. Unbound

cells were removed by flowing filtered PBS through the channel for 15 min at a flowrate of 100 ml/day.

A 1:200 dilution of SNAP-Cell 505-Star in PBS was added to the channel and incubated 30 min. Unbound fluorophores were removed by a 15 min PBS rinse. Cells were then induced by adding M9 media supplemented with arabinose to the channel with 24 h of incubation at room temperature. Once cells were fully induced, a 15 min PBS rinse was applied before SNAP-Cell 505-Star at 1:200 dilution was flowed into the channel again. After 30 min, a final PBS rinse (15 min) was applied to wash off all unbound dye.

## 3. Results

Our effort to engineer a living biomaterial surface was based on leveraging the tools of synthetic biology combined with robust surface chemistry and microfluidic technology. Our approach (Figure 1) required us to first create a microfluidic device consisting of a PDMS channel plasma-bonded to a glass substrate. This glass substrate was chemically primed (Figure 1a) using a multistep process allowing *E. coli* to covalently attach to the surface (Figure 1a). These *E. coli* harbored the synthetic construct (Figure 1b) developed as part of this work. Upon induction (Figure 1a and b), the surface-displayed construct was presented to the microfluidic environment, where the BG-modified species of interest could be sequestered by the living biomaterial from the bulk flow (Figure 1a and c). We could precisely drive the rate of flow in the system using a previously published, open-source microfluidic system we previously developed.<sup>43</sup> Results describing the function of each component of this system are described in the following sections.

### 3.1 Surface-displayed SNAP

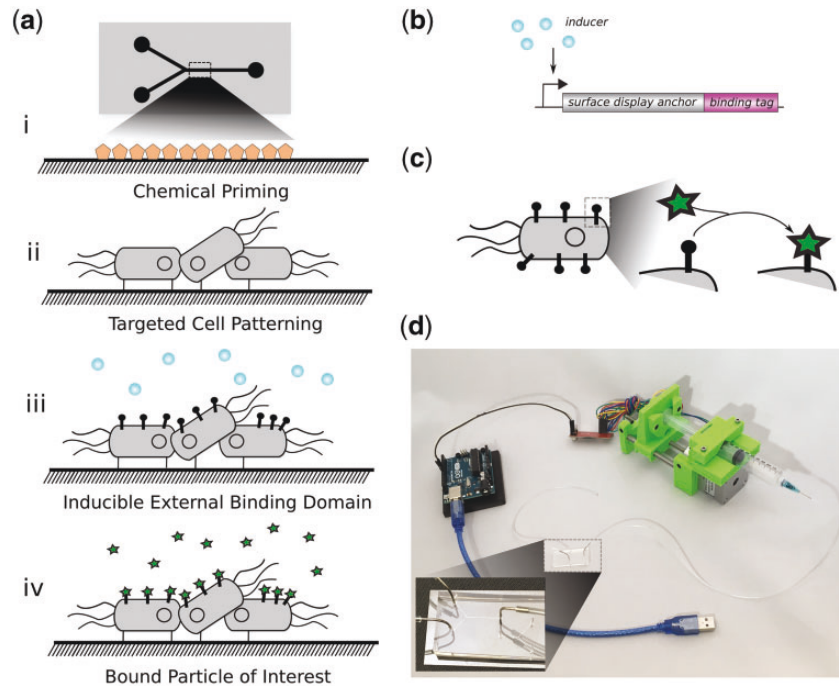
The mechanism of SNAP's function utilizes the covalent bonding of a BG-modified substrate to the thiol group in the active site of the SNAP protein. This bonding results in the release of a guanine group and the formation of a permanent covalent bond,<sup>44</sup> making the SNAP-system an ideal candidate for targeted molecular tagging.

The surface-displayed-SNAP plasmid (Supplementary Figure S1) was transformed into K-12 *E. coli* strain MG1655 $\Delta$ araBAD and induction studies were performed. After 6 h of growth with arabinose, cells were labelled using SNAP-Cell<sup>®</sup> 505-Star fluorescent label (Figure 2a). SNAP-Cell<sup>®</sup> 505-Star was an ideal candidate for testing surface-displayed SNAP because the label excitation and emission spectra do not result in significant cross-talk with mCherry and the label binding was restricted to the cell's surface, reducing the chance of labelling SNAP proteins present in the cytosol (Figure 2b). This inability of 505-Star to enter the cell helped to confirm the localization of the SNAP-protein to the cell's surface. Induced cells demonstrated a statistically significant shift in labelling affinity, measured through flow cytometry with a P-value of  $< 0.0001$  by ANOVA (Figure 2c and d). Furthermore, a dose responsive relationship was demonstrated (Figure 2e).

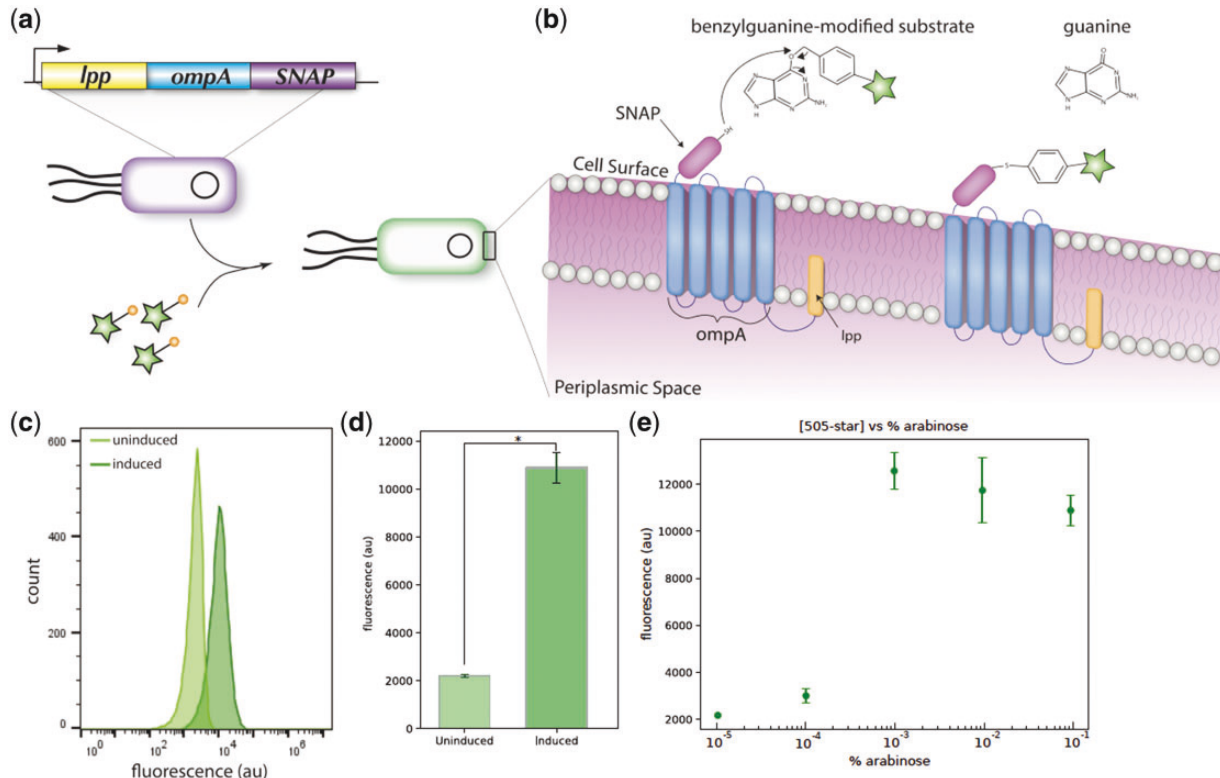
### 3.2 Spatially discretized fluorescence in the cytosol and at the cell surface

To fully demonstrate spatial discretization of protein location between the cytosol and cell surface, an mCherry gene was added to the construct (Figure 3a). To demonstrate simultaneous induction of both cytosolic and surface proteins, we replaced the constitutive promoter with a  $P_{\text{BAD}}$  promoter and

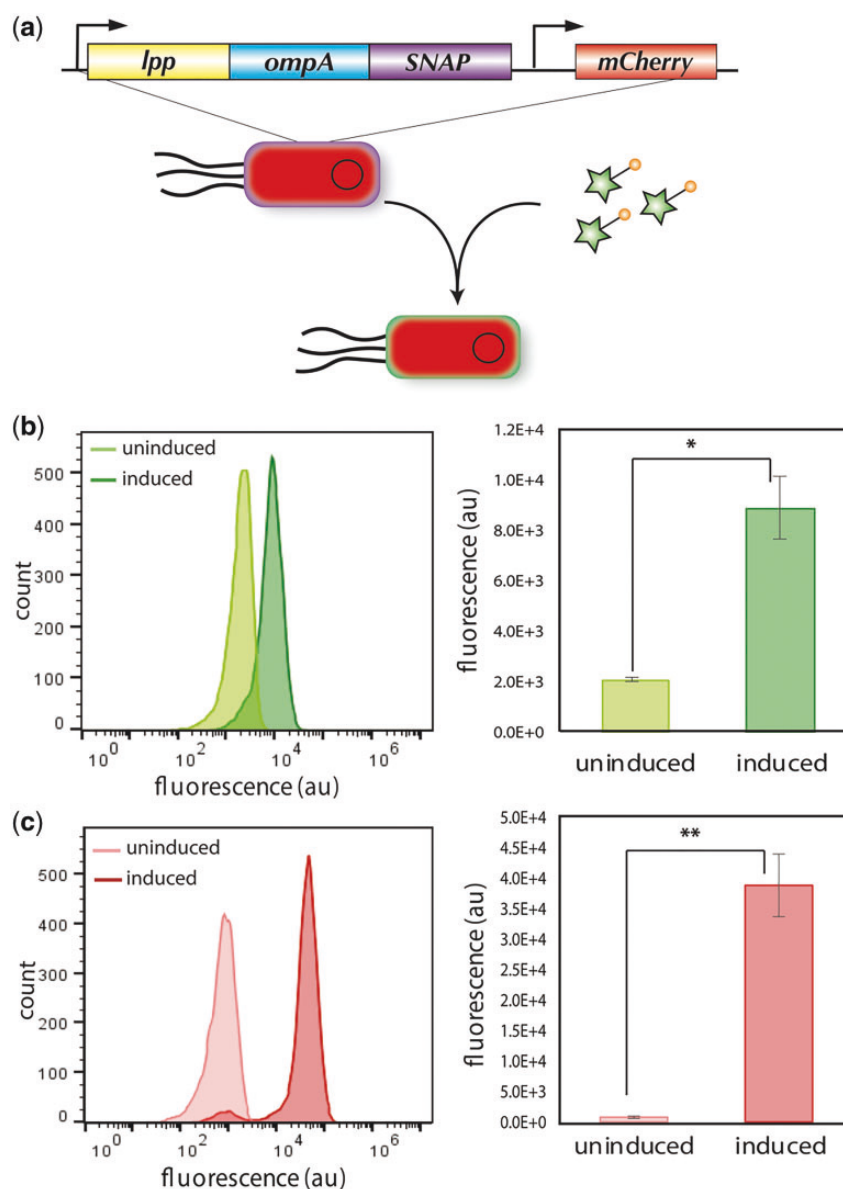




**Figure 1.** Inducible biomaterial synthesis exploiting spatially segregated genetic outputs. (a) A process for biomaterial formation: (i) a material surface such, as glass, is chemically primed to allow for cell adhesion. (ii) Engineered cells attach to the primed surface. (iii) Small molecule inducers are introduced to the cell microenvironment. (iv) BG-modified particles of interest are introduced, and covalently bond, to the surface of the living cells. (b) This system is enabled by engineering the cells to contain a genetic construct that encodes for the expression of a surface-displayed protein tag. (c) This tag allows for a covalent bond to form between cell-anchored protein structures and any BG-functionalized species of interest. (d) This strategy of biomaterial formation is enabled by precisely controlling the media and environment around the cells using custom-built pressure regulating syringe pumps previously reported.<sup>43</sup>



**Figure 2.** Examination of the functionality of SNAP was determined by using a fluorescent label modified with a BG group (SNAP-Cell<sup>®</sup> 505-Star). (a) Schematic of surface-display system and (b) fluorescent label addition. (c) Histogram and (d) bar graph from flow cytometry analysis showing the shift in fluorescence of cells labelled after growth with and without inducer. Tests were performed in triplicate with 10 000 events per sample (\**P* < 0.0001, *n* = 3). (e) Dose response curve of the labelled surface-displayed SNAP.



**Figure 3.** mCherry expression throughout the cytosol along with SNAP on the outer surface. (a) Gene schematic of the red cells labelled with a green fluorophore. (b) Histogram (left) and bar graph (right) of flow cytometry data showing induction of labelled on the cell surface. Tests were performed in triplicate with 10 000 events per sample (\* $P < 0.0001$ ). (c) Histogram (left) and bar graph (right) of flow cytometry data showing induction of mCherry throughout the cytosol. Tests were performed in triplicate with 10 000 events per sample (\*\* $P < 0.0001$ ,  $n = 3$ ).

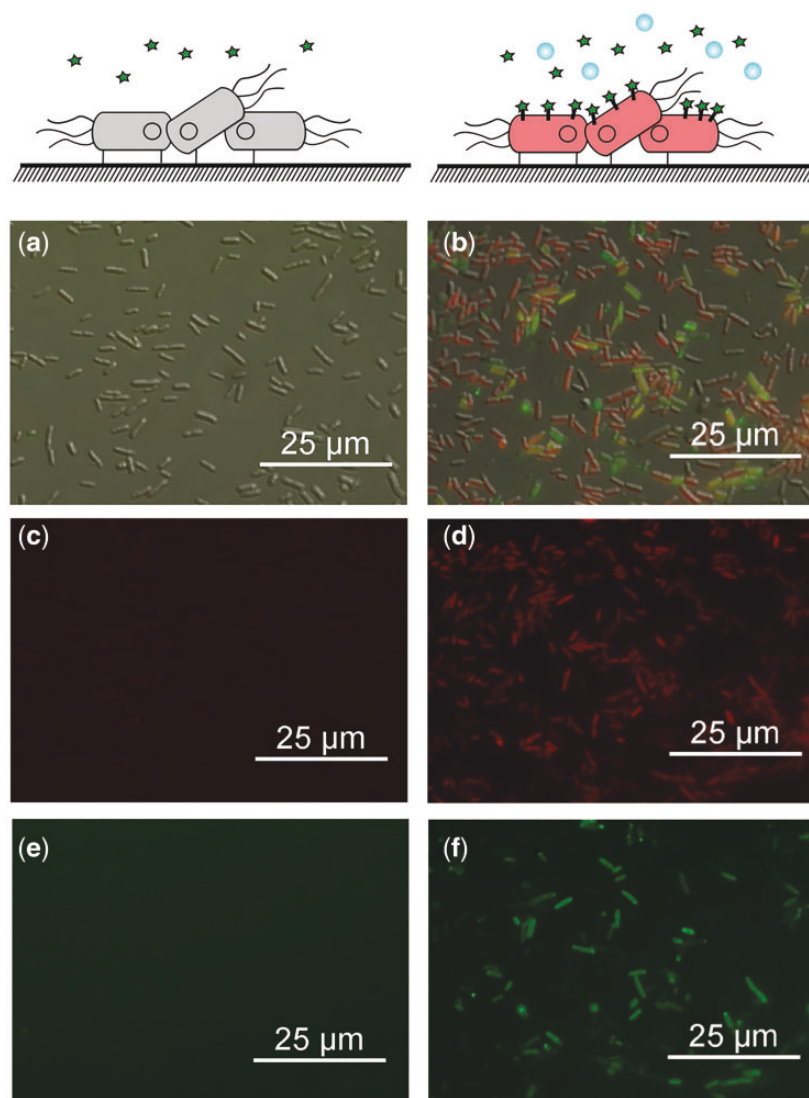
were able to achieve both cytosolic mCherry and *lpp-ompA*-SNAP protein expression (Supplementary Figures S2–S4) in induced cells, while un-induced cells did not produce mCherry and remained unlabeled (Figure 3b). Through statistical analysis, we demonstrated there was a significant increase between induced (0.1% arabinose) and un-induced (0% arabinose) cells with a  $P$ -value of  $< 0.0001$  by ANOVA (Figure 3c and d).

### 3.3 Bio-functionalization of a microfluidic device and small molecule binding

Cells containing the engineered plasmid, and thus capable of simultaneous expression of cytosolic mCherry and surface-displayed SNAP tag, were used to bio-functionalize the glass substrate in a microfluidic device to form a living biomaterial. This process began by activating the surface with hydroxyl groups through plasma

cleaning, silanizing with APTES and crosslinking the surface with GA. Introduction of *E. coli* allowed immobilization of cells on the reactive glass surface of the channel. Biomaterial formation was induced by first introducing the inducer arabinose into the microfluidic channel and then adding BG-functionalized molecules for material modification.

Automated syringe pumps<sup>43</sup> and fluorescent microscopy allowed us to confirm the formation of a complete biomaterial on the glass substrate within the microfluidic channel. Distinct shifts in the fluorescence characteristics of the patterned surface with un-induced and induced cells (Figure 4) indicated that the phenotypic responses to arabinose induction aligned with our strategy for biomaterial formation with induced cells showing both red fluorescence (cytosolic mCherry expression) and green fluorescence (sequestered BG-functionalized SNAP 505-star dye). These changes confirmed the creation of a living



**Figure 4.** Biomaterial formation and biomolecule sequestration within a microfluidic device. Three different channels are presented for a population of cells with and without arabinose. (a) In the absence of arabinose, cells still adhere to the microfluidic device. (c, e) However, the cells do not exhibit any fluorescence in the spectra corresponding with mCherry or 505-star. (b) Upon induction, cells begin to fluorescence red (d) through the expression of cytosolic mCherry, indicating the cell's ability to selectively bind molecules added to the system. (f) This functionality is visually confirmed by the addition of BG-modified 505-Star and increase in green fluorescence. In contrast, no red or green fluorescence is detected after the addition of BG-modified 505-star to un-induced cells.

biomaterial surface within a dynamic microfluidic environment. In this system, cells form a patterned living material that acts as both a chemical sensor for arabinose and a biomaterial nucleation point for additive material assembly as BG-modified molecules are bonded to the living surface. To elaborate, the patterned cells are able to sense arabinose, which in turn induces a genetic response that shifts the cell population's external chemistry. Thus, the engineered living cells function as a smart, adaptive biomaterial that can respond to, and bind with, small molecules from the local environment.

#### 4. Discussion

The creation of new biomaterials using the tools of synthetic biology has emerged as an important thrust in biomaterials engineering.<sup>26,45</sup> Many approaches have focused on engineering

living cells to synthesize one or more components of an eventual biomaterial.<sup>46,47</sup> Alternatively, cells can be programmed to produce molecules that interfere with biomaterial assembly.<sup>48</sup> In both these synthesis and interference approaches, the cell's ability to function as a molecular factory is leveraged. In other materials science approaches, cells themselves can be embedded as constituents within the biomaterial.<sup>49</sup>

In the results presented here, we have engineered a fluid-swept bacterial surface with an ability to bond to small molecules in its environment upon induction. These results describe a synthetic component, an *E. coli* surface-displayed SNAP<sup>®</sup> enzyme, along with an approach to using this component in microfluidic devices that demonstrates its use as a nucleation point for biomaterials assembly in microfluidic systems. Here, the surface-displayed enzyme bonded to BG-modified fluorescent dye molecules. However, straightforward chemistry and widely available BG-linkers allow a broad range of

macromolecules to be BG-linked. Thus, these results potentially can be leveraged by others for inducible sequestration of other BG-modified macromolecules.

Although this BG-modification requirement is a limitation of the system presented here, one advantage this system is its robust function under flow conditions in microfluidic devices. Other proteins can be potentially surface-displayed by modifying the synthetic construct reported here, potentially for extracellular chemical catalysis<sup>50</sup> or alternatively, as an antigen nucleation point for biomaterials assembly based on antibody-antigen interactions.<sup>51</sup>

Another limitation of this system is the random patterning of living cells in the microfluidic device. The primary focus of the work presented here is the demonstration of an extracellular genetic output that can interact with the cell's environment. Fortunately, multiple other studies have focused on different approaches to cellular patterning using synthetic biology.<sup>52,53</sup> These systems could potentially be combined to create patterned biomaterials with living constituents that bond to extracellular small molecules.

The field of synthetic biology has allowed for the programmable control of cellular behaviors through the use of genetic components and their interactions.<sup>54</sup> Most of these components are expressed either in the cytosol or, if cell-cell communication is involved, by the movement of signaling molecules out of the cell where neighboring cells may detect them using quorum-sensing strategies.<sup>17,55</sup> In contrast to these approaches, we used the cell's ability to spatially compartmentalize protein output to enable biomaterial formation. Leveraging synthetic behaviors compartmentalized on the cell surface allowed us to create a nucleation point for molecular assembly that is spatially separated from phenotypic changes in the cell's cytosol. Through this approach, we were able to form a biomaterial that can sense a small molecule using synthetic machinery in the cytosol while assembling a material using surface-displayed synthetic machinery.

Beyond biomaterial formation, this technology could be utilized for other synthetic biology applications. For example, by building upon the BG-binding membrane system, this platform could be used for cell sequestration of a range of species of interest. For example, if BG-functionalized antibodies were introduced to the fluid system, antigens that can be targeted with antibodies could be sequestered from the bulk flow. Furthermore, this tool could be adapted and deployed with cell-free expression technology, an area where there has been successful demonstration of the localization of expressed proteins to the surface of the vesicles,<sup>56</sup> as well as  $\alpha$ -hemolysin pore formation on the phospholipid bilayer.<sup>57</sup>

The work presented here can serve as an enabling technology for biomaterial synthesis and assembly. By engineering living cells that can sense, respond, and draw molecules from the local environment as the building blocks for a biomaterial, we experimentally validated a strategy for material formation using surface-displayed synthetic biology. Furthermore, by exploiting synthetic gene constructs that enable cytosolic sensing and surface-display-based material formation, we have shown how synthetic biology may leverage spatial compartmentalization for discrete functions in the same cell. We envision our living biomaterial being used in a range of applications from biomaterial formation to biomolecule sequestration.

## 5. Data availability

Data available at flowrepository.org ID FR-FCM-ZZTR (pFYS018) and FR-FCM-ZZTT (pFYS028); pFYS018 and pFYS028 nucleotide

sequences can be found with GenBank accession numbers KX904358, and KX904360, respectively.

## Supplementary data

Supplementary Data are available at SYNBIO Online.

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Conflict of interest statement. None declared.

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