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Living materials with programmable functionalities grown from engineered microbial co-cultures

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Biological systems assemble living materials that are autonomously patterned, can self-repair and can sense and respond to their environment. The field of engineered living materials aims to create novel materials with properties similar to those of natural biomaterials using genetically engineered organisms. Here, we describe an approach to fabricating functional bacterial cellulose-based living materials using a stable co-culture of *Saccharomyces cerevisiae* yeast and bacterial cellulose-producing *Komagataeibacter rhaeticus* bacteria. Yeast strains can be engineered to secrete enzymes into bacterial cellulose, generating autonomously grown catalytic materials and enabling DNA-encoded modification of bacterial cellulose bulk properties. Alternatively, engineered yeast can be incorporated within the growing cellulose matrix, creating living materials that can sense and respond to chemical and optical stimuli. This symbiotic culture of bacteria and yeast is a flexible platform for the production of bacterial cellulose-based engineered living materials with potential applications in biosensing and biocatalysis.

The nascent field of engineered living materials (ELMs) aims to recapitulate desirable properties of natural living biomaterials to create useful new materials using genetically engineered organisms[1–4]. Existing ELMs exhibit a remarkable range of functions but often require intensive manual processing steps for purification, functionalization or patterning[5–14]. Others make use of the multiple functionalities of living cells by embedding them within synthetic hydrogels[15–20]. However, a long-term goal of ELMs research is to use engineered cells, rationally reprogrammed with DNA, to simultaneously make the material and incorporate novel functionalities into it—thus 'growing' functional biomaterials in situ[3].

Natural living materials often rely on division of labour between specialized cells performing particular functions to achieve such self-assembly. Plant leaves, for example, are self-assembling living materials in which specialized cells are responsible for different traits (Fig. 1a). Inspired by this, we sought to develop a microbial ELM that utilized a similar approach, dividing bulk material production and functional modification between co-cultured cell types suited for each specialism and thus enabling complex ELMs to be self-assembled in situ.

Bacterial cellulose (BC) has recently emerged as a promising bulk material for ELM production due to high production yields that achieve >10 grams per litre from just a few days' growth in low cost, simple sugar media[21]. Various species of Gram-negative acetic acid bacteria, particularly members of the *Komagataeibacter* and *Gluconacetobacter* genera, produce high quantities of extracellular cellulose. When grown in shallow trays containing static cultures, they yield continuous BC sheets with large surface areas (>1,000 cm^2)[22]. Numerous individual glucan chains are first secreted, then bundled into cellulose fibrils. Over several days, a thick floating mat called a pellicle forms, consisting of a network of BC fibrils around embedded BC-producing bacteria. The resulting material (Fig. 1b) is a dense network of ribbon-like cellulose fibrils, each ~50 nm wide and up to 9 µm in length, held together tightly by van der Waals forces and hydrogen bonds[22].

The ultrapure nature and high crystallinity of BC afford excellent mechanical properties, with individual nanofibres estimated to have tensile strengths of at least 2 GPa and Young's moduli of ~138 GPa[23,24]. BC has high porosity, high water retention and a very large surface area. It is both biodegradable and biocompatible and can be made at scale with minimal equipment and low environmental impact and cost. Consequently, BC-based materials have attracted interest for use as surgical and wound dressings, acoustic diaphragms for headphones and speakers, battery separators, additives to cosmetics and scaffolds for tissue engineering[25]. Several BC-based materials have been commercially developed for medical and cosmetic applications (for example, as wound dressings for the treatment of burns and ulcers)[26].

Genetic modification of BC-producing bacteria has previously been used to alter BC material properties by, for instance, generating non-native chitin-cellulose[27] and curdlan-cellulose[28] co-polymer materials or optimizing cellulose yields to enable production of battery separators[29]. Modular genetic toolkits for engineering BC-producing bacteria have also been developed but remain minimal in comparison to those for model organisms[30–33]. Crucially, there is a lack of genetic tools and knowledge to enable recombinant protein secretion from BC-producing bacteria, and their ability to be reprogrammed to sense external cues is also poor.

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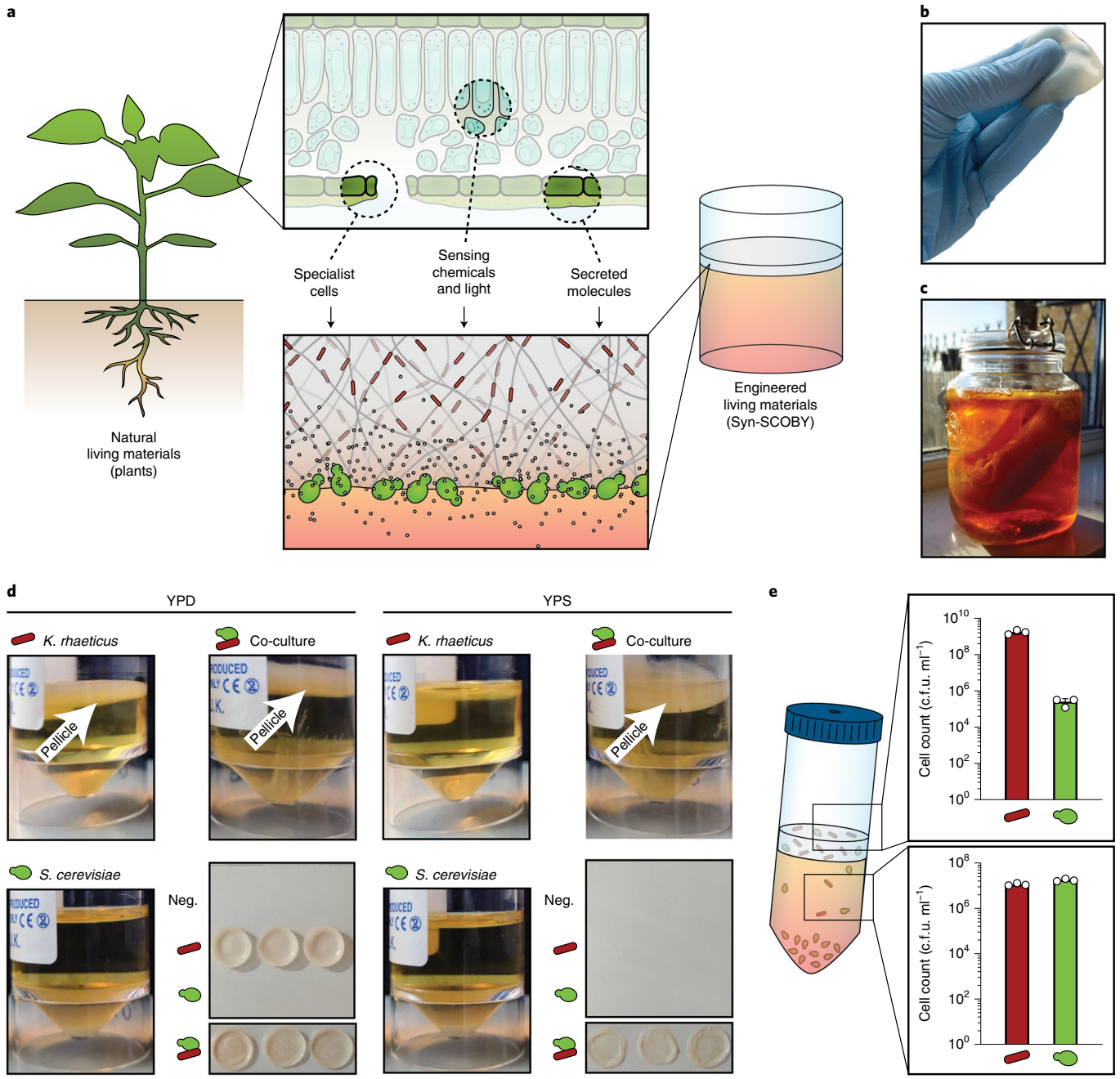


Fig. 1 | Generating Syn-SCOBY co-cultures with *S. cerevisiae* and *K. rhaeticus*. **a**, Schematic showing analogies between natural living materials (plants) and engineered living Syn-SCOBY materials. Yeast cells are depicted in green and bacteria are depicted in red. **b**, An image of a BC pellicle, a flexible but tough material. **c**, Home-brewed kombucha tea. Both a large submerged mass of BC from previous rounds of fermentation as well as a newly formed thinner layer at the surface can be seen. **d**, Images of monocultures and co-cultures of *K. rhaeticus* and *S. cerevisiae* grown in either YPS or YPD medium for 3 days. Dense *S. cerevisiae* growth can be seen as a sediment at the base of the culture. *K. rhaeticus* growth results in a thick pellicle layer at the air–water interface (white arrow). The lower right panels show the harvetsted pellicles from three independent experiments. **e**, Cell counts of a co-culture consisting of *K. rhaeticus* Kr RFP and *S. cerevisiae* yWS167 strains grown in YPS media. Cell counts were determined by plating and counting the numbers of cells present in the two phases of the co-cultures, the liquid layer and the pellicle layer. Samples were prepared in triplicate; error bars represent ±1 s.d.

These factors severely limit the engineerability and versatility of BC-based ELMs.

To create an ELM system that leverages specialist engineered cells among bulk-producer cells, we took inspiration from kombucha tea. Kombucha is a fermented beverage produced by a microbial community commonly referred to as a symbiotic culture of bacteria and yeast (SCOBY) (Fig. 1c). SCOBYs typically consist of BC-producing bacteria and at least one species of yeast. Notably, one of the yeast species often found in kombucha fermentations is the yeast model organism *S. cerevisiae*[34].

Here we recreate co-cultures of a BC-producing bacterium, *K. rhaeticus*[30], with engineered lab strains of *S. cerevisiae* in order to develop a novel ELM system that we call a synthetic SCOBY (Syn-SCOBY). Stable co-cultivation of yeast during the cellulose

biofilm production phase provides a host cell that can easily be rationally programmed with DNA for dedicated tasks (Fig. 1a). Engineered *S. cerevisiae* act as specialist cells in the system, secreting proteins, sensing chemical and physical signals and modifying the material properties of the surrounding cellulose. In light of the goals of ELMs research, we show that all of these functionalities can be achieved in Syn-SCOBY materials that grow and self-assemble in their entirety from only simple growth media in a few days.

Establishing conditions for stable Syn-SCOBY growth

To introduce yeast as specialist cells within a bacterial culture used for BC material production, we first identified conditions in which *K. rhaeticus* and *S. cerevisiae* can be efficiently co-cultured. This required screening for growth at a range of inoculation ratios and in different growth media (Supplementary text). An initial screen revealed that *K. rhaeticus* grew poorly in sucrose-containing media compared to glucose-containing media, failing to form a pellicle after 3 days (Fig. 1d and Supplementary Fig. 1). However, co-culturing *K. rhaeticus* with *S. cerevisiae* in the same sucrose media enabled growth and pellicle formation, leading to our Syn-SCOBY co-culture protocol, in which BC formation was dependent on the continued presence of the yeast (Supplementary Fig. 2). Under these conditions, the majority of *K. rhaeticus* cells were found in the pellicle layer, while the majority of *S. cerevisiae* cells were found in the liquid layer (Fig. 1e). We characterized a range of other co-culture parameters, including pellicle yields, co-culture stability, cell distribution and reproducibility (Supplementary text and Supplementary Figs. 3–8). Notably, we provide evidence that the growth enhancement conferred by yeast is based on secretion of invertase enzyme, which hydrolyses sucrose to monosaccharide sugars that are more efficiently metabolized by the bacteria (Supplementary Fig. 6).

Engineering Syn-SCOBY yeast to functionalize BC

Having developed a robust *S. cerevisiae–K. rhaeticus* co-culture, we set out to engineer *S. cerevisiae* to act as a specialist cell conferring novel functional properties (Fig. 2a). Unlike BC-producing bacteria, yeasts have a high capacity for secretion of recombinant proteins. Therefore, the presence of yeast in Syn-SCOBY co-cultures offers a route to BC functionalization by engineering *S. cerevisiae* strains to secrete proteins that form part of grown BC materials (Fig. 2b). We assessed this capability with the β-lactam hydrolysing enzyme TEM1 β-lactamase (BLA). Using the yeast toolkit (YTK) system for modular genetic engineering[35], the BLA catalytic region was cloned downstream of the mating factor alpha (MFα) secretion signal peptide under the control of a strong constitutive promoter to create yeast strain yCG04 (Fig. 2c). However, as the pellicle makes up only a part of the co-culture volume, we hypothesized that fusion of a cellulose-binding domain (CBD) to this enzyme might increase the proportion of secreted protein incorporated within the BC layer. Therefore, a second strain (yCG05) was engineered in which a CBD was fused to the C terminus of the BLA protein (Fig. 2c). The 112 amino acid region from the C terminus of the Cex exoglucanase from *Cellulomonas fimi* (CBDCex)[36] was chosen based on previous work demonstrating its ability to bind BC[30]. Monocultures of yCG04 and yCG05 were screened for BLA activity using the colorimetric nitrocefin assay (Fig. 2d), confirming secretion of active BLA and BLA-CBD, respectively (Supplementary Fig. 9). Next, co-cultures of wild-type (WT) yCG04 or yCG05 *S. cerevisiae* strains with *K. rhaeticus* bacteria were grown and the resultant BC pellicles were screened for BLA activity. While pellicles grown with WT yeast showed no BLA activity, clear activity was observed from pellicles co-cultured with BLA-secreting and BLA-CBD-secreting strains (Fig. 2e), demonstrating direct BC functionalization. Notably, fusion of the CBD to the enzyme resulted in an increase in observed BLA signal from the BC samples. In addition, BLA-CBD functionalized pellicles appeared to retain catalytic activity more strongly after washing than those with only BLA (Supplementary Fig. 10). Whether these observations originate from a difference in the number of bound BLA and BLA-CBD proteins in the material requires further exploration.

We then tested whether enzyme-functionalized BC that had been dried and rehydrated retained catalytic activity. Pellicles produced by co-culturing were dried to create thin, paper-like materials (Supplementary Fig. 11) and later rehydrated and assayed for BLA activity. Rehydrated pellicles functionalized with both BLA and BLA-CBD demonstrated clear activity (Fig. 2f). For absolute quantification of BLA activity, assays were run with pellicles derived from co-cultures with WT yeast spiked with known concentrations of commercial BLA enzyme to create standard curves (Supplementary Fig. 12). This revealed that the drying process itself had little effect on the BLA activity of the material, which was $29.8 \pm 3.7\ \mathrm{mU\,cm^{-2}}$ before drying and $27.3 \pm 4.4\ \mathrm{mU\,cm^{-2}}$ after (Fig. 2g). Identical assays were performed following storage of materials for 1 month or 6 months at room temperature without desiccant. After storage, enzymatic activity was retained, although it was reduced to one-third the original level (Fig. 2g). Overall, these experiments show that functionalized materials can be grown and stored at room temperature, retaining their activity for later rehydration and deployment.

To demonstrate the generalizability of our approach, we generated BC functionalized with two other enzymes: an α-galactosidase (Mel1) and a laccase from *Coriolopsis trogii* (CtLcc1)[37] (Supplementary text and Supplementary Figs. 13 and 14). Yeast secreting these enzymes were co-cultured with *K. rhaeticus* and the resultant pellicles assayed for enzyme activity. Laccase and α-galactosidase activities were detected in native, wet pellicles and were retained after drying and rehydration (Fig. 2i,k).

The Syn-SCOBY approach enables self-assembly of enzyme-functionalized BC materials grown under mild conditions from simple raw materials. Importantly, these materials retain activity following drying and rehydration after storage under ambient conditions, suggesting this approach could be applied to the production of immobilized enzyme materials to be used in various industrial processes (Supplementary text).

Modifying BC physical properties via enzyme secretion

The presence of living cells within ELMs would allow cells to be programmed to modify the physical properties of the material as it is grown or used. To enable this, we developed a method that increases the number of yeast cells incorporated into the BC pellicle by ~340-fold through tuning the density of the Syn-SCOBY culture medium with an inert culture medium additive called OptiPrep (Supplementary text and Supplementary Figs. 15–22).

Using this protocol, we engineered cellulase secretion from yeast to attempt to modify the bulk physical properties of the Syn-SCOBY BC. We constructed a yeast strain, yCelMix, in which cellobiohydrolases (CBH1 and CBH2), endoglucanase (EGL2), β-glucosidase (BGL1), and lytic polysaccharide monooxygenases (LPMO) are optimized to be secreted simultaneously for synergistic cellulose degradation, as described previously[38,39] (Fig. 3a,b and Supplementary Fig. 23). Co-cultures of *K. rhaeticus* and yCelMix yeast formed pellicles after 2 days, albeit with slightly decreased dried weight (Fig. 3c), indicating that rates of cellulase secretion and action were slower than the rate of BC biosynthesis. Extending incubation to 5 days did not further reduce the BC mass, potentially due to the limited diffusion of enzymes, low pH, local accumulation of reaction products or cellulase deactivation at the air–liquid interface in the static culture[40]. Cellulase secretion did not enhance leakage of yeast cells from pellicles but resulted in a smaller total surface area and changes in the fibre network packing (Supplementary text and Supplementary Figs. 24–26).

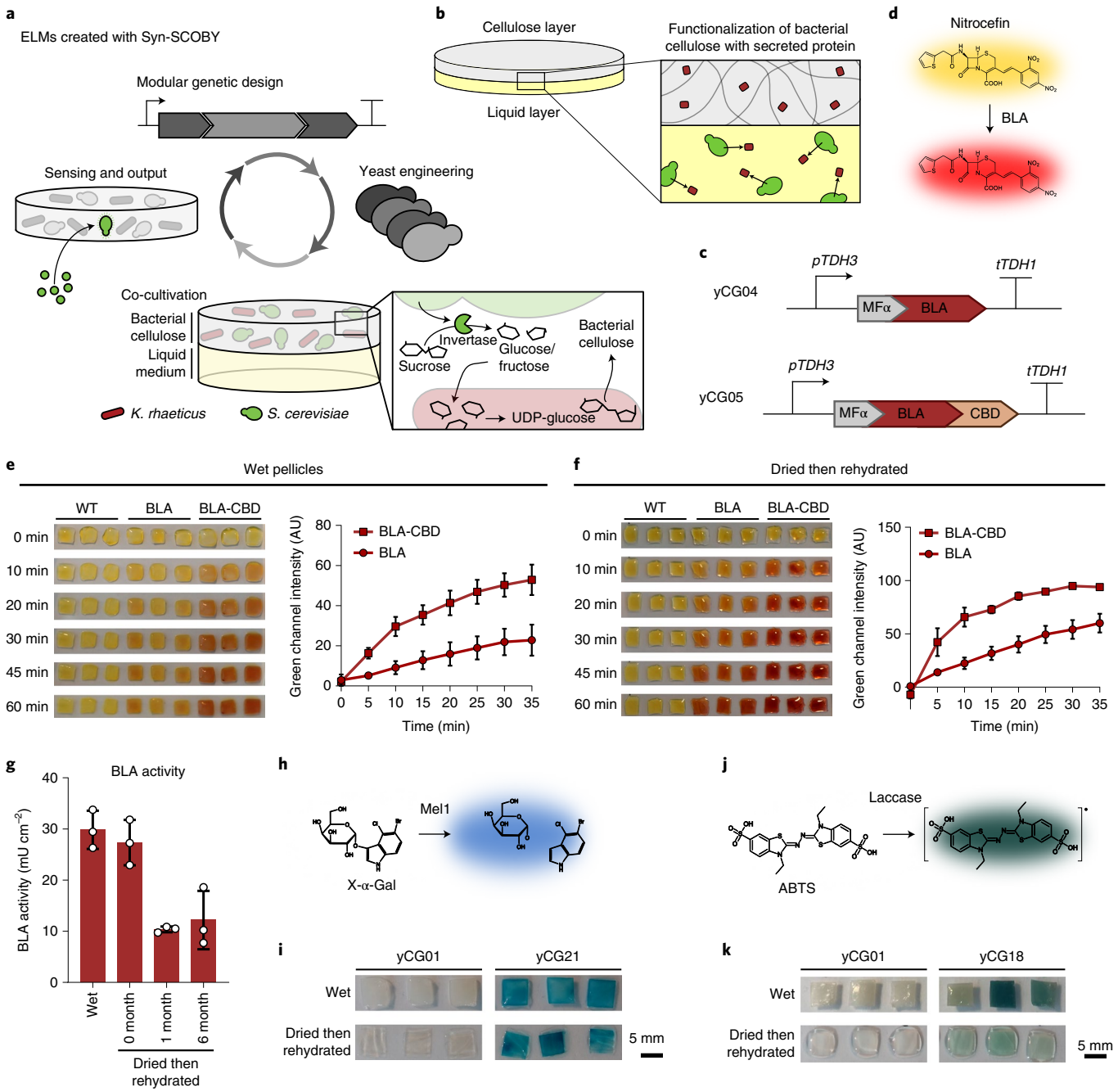


Fig. 2 | Syn-SCOBYs can produce enzyme-functionalized BC materials. a, Schematic summary of Syn-SCOBY ELMs (UDP = uridine diphosphate). **b**, Schematic illustrating concept of functionalization. *S. cerevisiae* cells (green) secrete a protein (red) that incorporates into the BC layer (grey). **c**, Genetic design of BLA-secreting yeast strains yCG04 (BLA) and yCG05 (BLA-CBD). **d**, Nitrocefin is converted from a yellow substrate to a red product by BLA. **e**,**f**, The nitrocefin assay was performed with cut 5 × 5 mm native wet pellicle samples (**e**) or with dried then rehydrated pellicle samples (**f**). In both cases co-cultures were prepared with *S. cerevisiae* BY4741 (WT), yCG04 (BLA) and yCG05 (BLA-CBD). Images are of pellicles after the indicated time points during the assay. The yellow-to-red colour change was quantified and plotted. Samples were prepared in triplicate; error bars represent ±1 s.d. **g**, Absolute BLA activities were calculated from native hydrated pellicles (wet) and from pellicles dried then rehydrated after the indicated time periods. Samples presented here are from pellicles grown in co-culture with yCG05. As pellicle liquid volume is altered by drying, BLA activity is represented by enzyme activity units per unit of pellicle area. Samples were prepared in triplicate; error bars represent ±1 s.d. **h**, X-α-Gal is converted from a colourless substrate to a blue product by the Mel1 enzyme. **i**, The X-α-Gal assay was performed with wet and dried then rehydrated pellicle samples from co-cultures with the GFP-secreting strain yCG01 or a strain that secretes Mel1 (yCG21). Samples were prepared in triplicate. **j**, ABTS is converted from a colourless substrate to a green product by laccase enzyme. **k**, The ABTS assay was performed with wet and dried then rehydrated pellicle samples from co-cultures with the GFP-secreting strain yCG01 or a strain that secretes CtLcc1 (yCG18). Samples were prepared in triplicate.

Although BC yield was not reduced by cellulase secretion, the mechanical properties of the pellicles were altered. Stress–strain curves from tensile tests demonstrated a clear difference between WT pellicles and yCelMix pellicles (Fig. 3d). Specifically, while both were brittle (as determined by fracture strength), the yCelMix pellicle could only sustain 45.7 MPa while the WT pellicle can bear

98.3 MPa of stress before fracturing (Fig. 3e). The yCelMix pellicle could also only be stretched to half the length that the WT pellicle could be elongated to before breaking (Fig. 3f). Furthermore, secreted cellulases reduced the stiffness of the matrix, lowering the Young's modulus from 7.2 GPa (WT) to 5.1 GPa (yCelMix) (Fig. 3g and Supplementary Fig. 27). Although the underlying mechanisms by which these enzymes modify the material properties require further investigation, our results are consistent with others who report that the continuity of the cellulose fibrils and integrity of network structure are essential for the strength and stiffness of BC materials[41,42].

The weakening of microstructure in the yCelMix pellicle was also reflected by its rheological properties. In strain sweep experiments (Fig. 3h), both pellicles showed gel-like behaviour at low strain, as G' always dominates over G''. The G' and G'' of yCelMix pellicle were both lower than those of WT pellicles, confirming that cellulases reduced the stiffness of the BC material (Fig. 3h) in a frequency-independent fashion (Fig. 3i). As the applied strain increased beyond the G'–G'' crossover point, where microcracks accumulated and major rupture appeared, the pellicles switched to a viscoelastic liquid and started to flow. Notably, the yCelMix pellicle had an earlier onset of crossover, indicating a faster breakdown of network structure in the matrix. The more pronounced G'' maximum in the yCelMix pellicle (Fig. 3i) also demonstrates that deformation energy was converted into friction heat from the free broken fibrils near the microcracks, which originated from a disintegrated and weaker cellulose network. Our results suggest cellulases secreted from the yeast can weaken the mechanical and viscoelastic properties of BC materials, complementing recent studies showing that cellulose-binding domain (CBD) protein additives can enhance the strength of cellulosic materials[43,44].

Engineering living materials to sense and respond

S. cerevisiae strains are routinely engineered in synthetic biology as biosensors that sense external stimuli and respond with changes in gene expression. Our modular co-culture Syn-SCOBY approach enables easy incorporation of biosensor yeast strains into grown BC materials (Fig. 4a). We selected a chemically inducible system where the oestrogen steroid hormone β-oestradiol (BED) triggers activation of transcription from a target promoter[45,46]. Specifically, we used an *S. cerevisiae* strain (yGPH093) expressing the BED-activated synthetic transcription factor Z_3EV and a green fluorescent protein (GFP) reporter under control of the Z_3EV-responsive promoter (Fig. 4b)[47]. Pellicles produced by *K. rhaeticus*–yGPH093 co-cultures generated a strong GFP signal when exposed to exogenous BED (Fig. 4c), demonstrating that the Syn-SCOBY approach can produce living BC materials that sense and respond to environmental stimuli.

To test the viability of Syn-SCOBY living materials after drying and long-term storage (Fig. 4d), pellicles containing a GFP-expressing yeast were grown from co-culture, dried and stored at room temperature. They were then degraded enzymatically, and viable cell counts of *S. cerevisiae* were determined by plating. Although drying resulted in a substantial loss of viable yeast within the BC, viable cells could be recovered even after 1 month of storage (Supplementary Fig. 28).

To demonstrate that the remaining viable cells were still sufficient for biosensor materials to remain functional, pellicles grown with yGPH093 were dried, stored and revived by incubating in fresh media for 24 h in the presence or absence of BED. Rehydrated pellicles containing yGPH093 once again yielded a clear GFP signal in the presence of BED (Fig. 4e), even after ambient storage of dried pellicles for 4 months (Fig. 4f). While these sense-and-response functions require addition of growth media, diverse sample types could be used by supplementing with concentrated nutrient stocks. A similar approach has previously enabled *S. cerevisiae* biosensor strains to function in blood, urine and soil[48]. As a demonstration of further biosensor capabilities, we grew and verified a BC sensor where engineered yeast sense a protein via a G-protein-coupled receptor (Supplementary text and Supplementary Fig. 29).

A goal of ELMs research is to generate materials that are dynamic, adapting in response to environmental changes. To demonstrate a step towards this, we linked yeast biosensors to the production of a functional response. Since laccase enzymes have been previously shown to degrade BED[49], we engineered yeast strain yCG23 to secrete laccase from the BED-inducible promoter (Fig. 4g and Supplementary Fig. 30). Co-culture of *K. rhaeticus* and yCG23 produced a BC-based living material that could detect the presence of BED and secrete active laccase enzyme in response (Fig. 4h). Notably, BED is an important environmental pollutant, with potential effects on exposed aquatic species and humans[50,51]. The Syn-SCOBY approach can therefore be used to grow materials programmed to sense changes in their environment and respond accordingly.

Spatial patterning of living materials by optogenetics

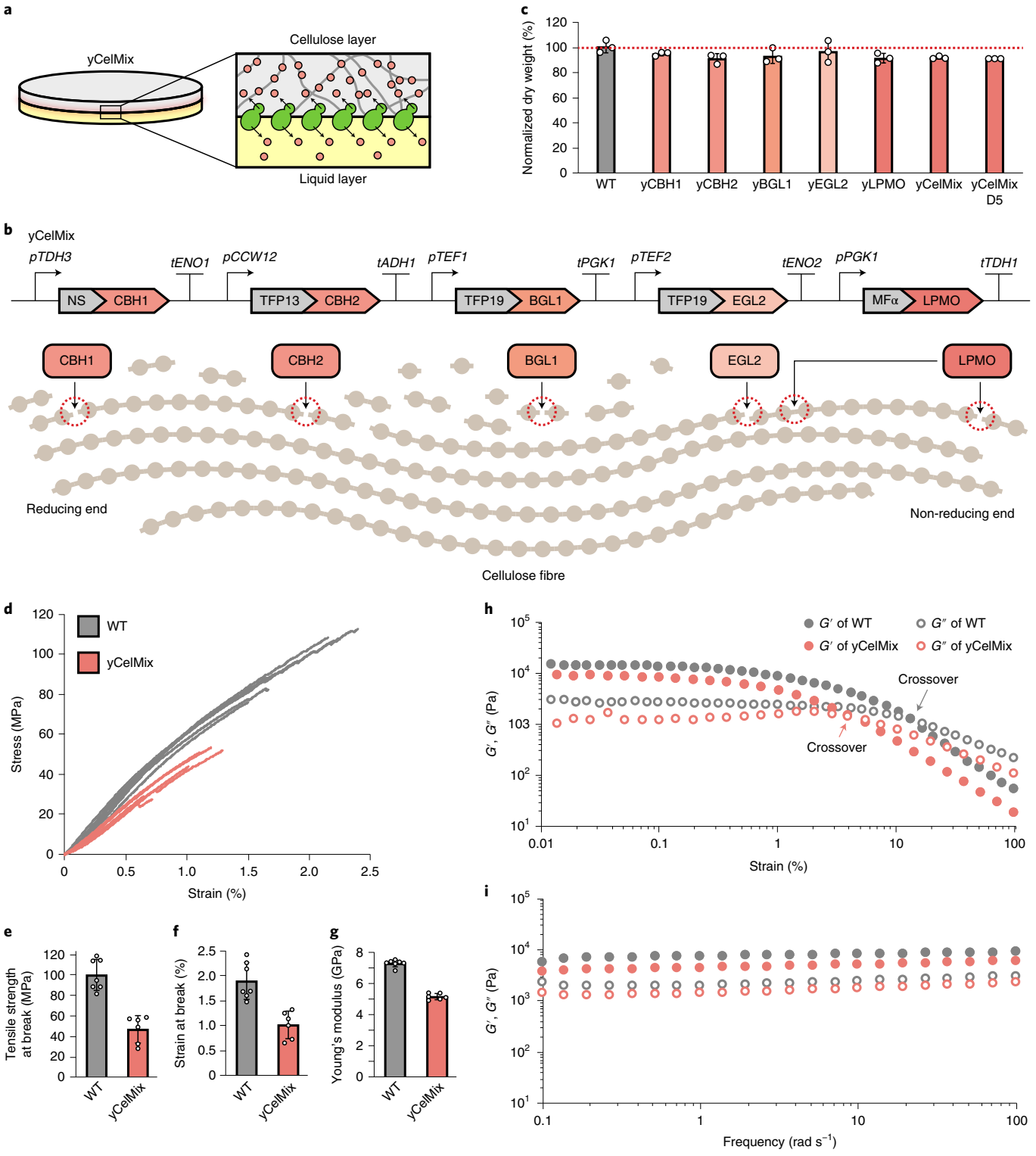
As an alternative to sensing chemical inputs, we used optogenetics to develop light-responsive BC-based ELMs. We implemented and optimized a blue-light sensing system in *S. cerevisiae* based on the CRY2–CIB transcription system[52] (Supplementary text and Supplementary Figs. 31 and 32a). The light-inducible promoter was engineered to trigger expression of NanoLuc, a luciferase reporter enzyme. Two versions were designed, a yNSurface strain that displays NanoLuc on the yeast cell surface, and yNCellulose, which secretes a NanoLuc-CBD version into the culture to bind to BC (Fig. 5a,b).

To demonstrate that Syn-SCOBY pellicles can be engineered to respond to an optical input, *K. rhaeticus* was co-cultured with each strain with or without exposure to white light. After 3 days

Fig. 3 | Modifying BC physical material properties. a, Schematic showing yCelMix cells secreting cellulases into the surrounding microenvironment from the bottom surface of the pellicle. **b**, Schematic illustrating the architecture of the yCelMix cellulase secretion strain. Expression of each cellulase is controlled by a distinct combination of strong promoter and terminator to prevent homologous recombination. CBH1 (cellobiohydrolase from *Chaetomium thermophilum*), CBH2 (cellobiohydrolase from *Chrysosporium lucknowense*), BGL1 (β-glucosidase from *Saccharomycopsis fibuligera*), and EGL2 (endoglucanase from *Trichoderma reesei*) are fused with optimal secretion signals as determined by Lee et al.[39] while LPMO (LPMO9H from *Podospora anserina*[38]) was fused with *S. cerevisiae* MFα signal peptide. Signal peptides are coloured grey (NS, native signal sequence; TFP13, translational fusion partner 13; TFP19, translational fusion partner 19). Cleavage sites for the cellulases on the cellulose polymer are marked as red circles. Each circle represents a monosaccharide unit in the cellulose polymer. **c**, Normalized pellicle dried weight after 2 day growth of *K. rhaeticus* with different cellulase-secreting *S. cerevisiae*. Red line is weight of WT yeast pellicle. yCelMix D5 is after 5 day growth. Samples prepared in triplicate; error bars represent ±1 s.d. **d**, Stress–strain curves of dried WT and yCelMix pellicles. Samples are from seven WT and six yCelMix co-cultures. **e**–**g**, Tensile strength at break, strain at break and Young's modulus calculated from the data in (**d**). Error bars represent ±1 s.d. **h**,**i**, Rheological properties of WT and yCelMix pellicles measured by strain sweep at 1 rad s^{-1} (**h**) and frequency sweep at 1% strain (**i**). Arrows in (**i**) indicate the crossover of the storage modulus (G', elastic deformation) and loss modulus (G'', viscous deformation).

of incubation in light, BC pellicles grown from both co-cultures formed material layers that exhibited high bioluminescence. Equivalent pellicles grown in the dark for 3 days showed nearly zero luciferase activity (Fig. 5c). The yNCellulose pellicle exhibited evenly distributed NanoLuc activity across the material surface, whereas the yNSurface pellicle had localized foci corresponding to the yeast distribution. This demonstrates that the local distribution of protein functionalization in BC materials can be genetically programmed into the co-cultured yeast.

We further explored the responsiveness of these pellicles to light patterns created by masking and projecting. With Syn-SCOBYs containing either yNSurface or yNCellulose yeast, we grew 'living films' in the dark for 3 days before further growth with light exposure (Fig. 5d). With light patterning by masking, both BC pellicles exhibited bright foci within their patterns, likely due to insufficient time for NanoLuc to diffuse away from yeast in this experiment. When the pattern was instead projected, the projected patterns were closely reproduced in the final luciferase activity output



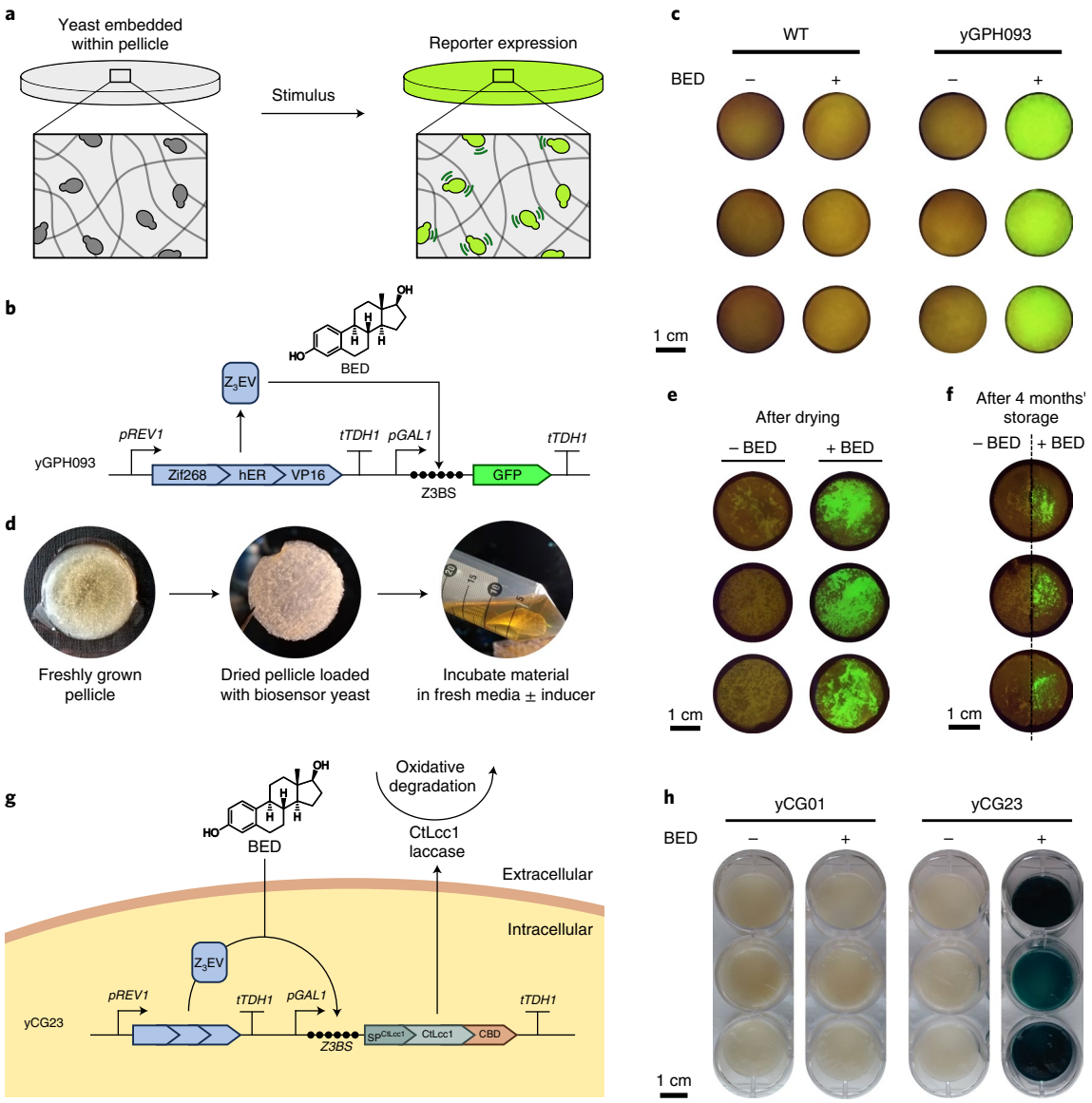


Fig. 4 | Syn-SCOBY materials can sense and respond. a, Schematic illustrating sense-and-respond pellicle function. Pellicles were grown containing engineered *S. cerevisiae* capable of detecting environmental stimuli and responding by gene expression. **b**, Schematic showing genetic yGPH093 circuit. The engineered *S. cerevisiae* (yGPH093) senses the presence of the chemical inducer BED and in response, produces the reporter protein GFP. The Z_3EV synthetic transcription factor is expressed from constitutive promoter *pREV1*. On addition of BED Z_3EV binds Z_3EV binding sites (*Z3BSs*) in the *pGAL1* promoter activating GFP expression. **c**, Testing biosensor pellicles. Pellicles were grown with either BY4741 (WT) or BED-responsive (yGPH093) yeast incorporated within the BC matrix. Triplicate samples were incubated with or without BED and imaged for GFP fluorescence by transilluminator after 24 h. **d**, Pellicles into which *S. cerevisiae* has been incorporated can be dried, stored and then revived by incubating in fresh media with or without inducer. **e**,**f**, Dried pellicles into which yGPH093 had been incorporated were incubated in fresh media in the presence or absence of BED following ambient storage for 1 day (**e**) or 4 months (**f**). After 24 h, pellicles were imaged for GFP fluorescence by transilluminator. Samples were prepared in triplicate. **g**, Schematic illustrating yCG23 construct design. Similar to yGPH093, yCG23 enables BED-inducible expression of secreted CtLcc1 laccase, directed by its native signal peptide (SP^{CtLcc1}). Extracellular laccases can then be used to oxidize a broad range of substrates, enabling degradation of environmental pollutants. **h**, Native wet pellicles from YPS+OptiPrep co-cultures of the GFP-secreting yCG01 strain and yCG23 were harvested, washed and inoculated into fresh medium with or without BED. After 24 hours of growth, pellicles were again harvested and washed and assayed for laccase activity using the colorimetric ABTS assay. Samples were prepared in triplicate.

(Fig. 5e). Here, yNSurface produced a sharper pattern compared with yNCellulose, which is expected due to the ability of NanoLuc to diffuse in the latter case.

We anticipate that this system could be readily expandable to multicolour optogenetics by further incorporating alternative light-based dimerizing systems[52] and linking these to other enzymatic outputs, enabling spatially segregated enzymatic cascade in BC materials.

Outlook

BC materials are biocompatible, produced by growth under mild conditions with simple culture media and made in high yield at little cost from microbes already commonly used in both the food and healthcare industries. As such, BC is a promising biological material for the development of ELMs. Our Syn-SCOBY approach represents a major improvement in the engineerability of BC, bringing many potential applications into scope.

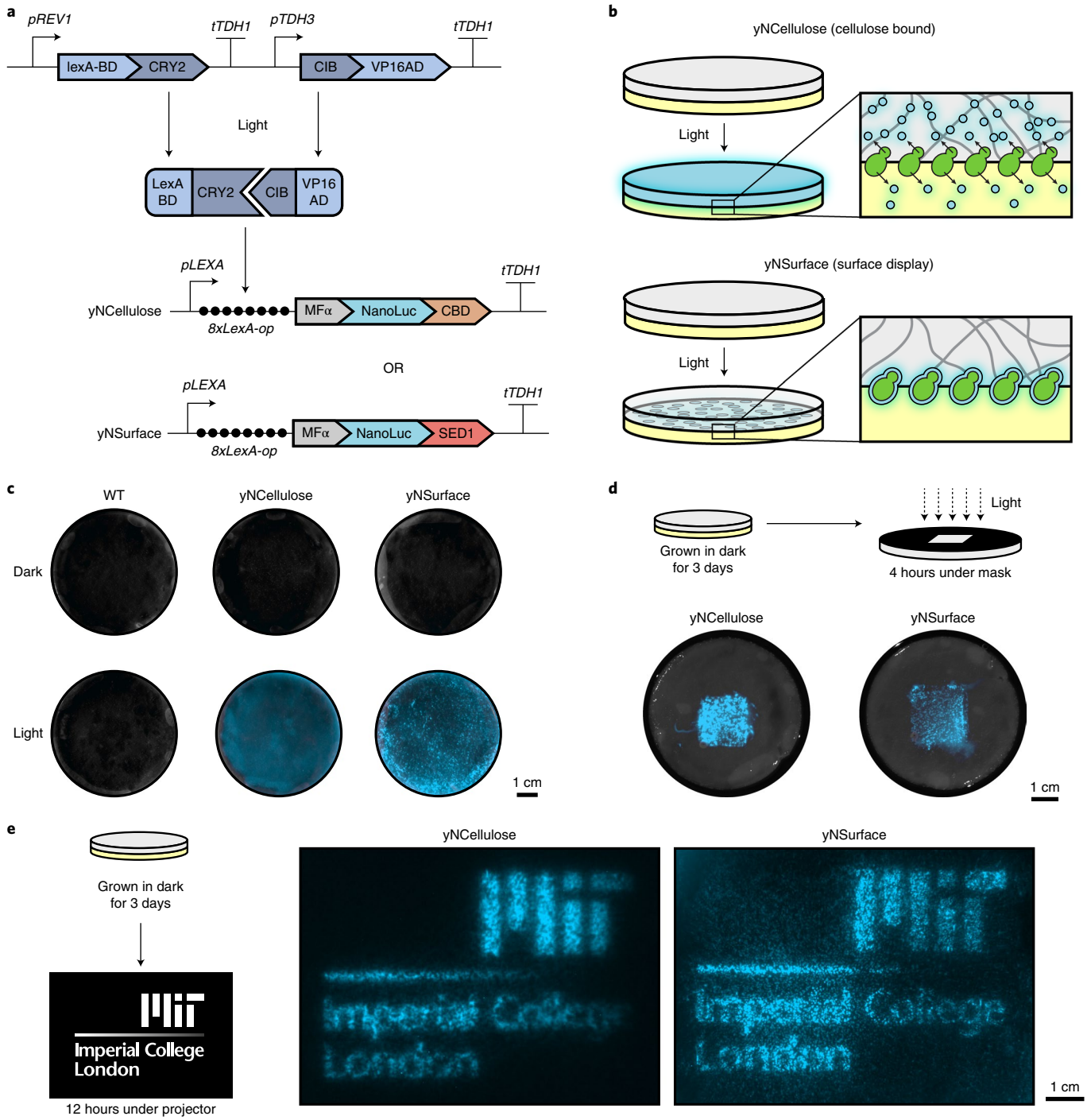


Fig. 5 | Optical patterning of enzymatically functionalized BC materials. a, Schematic showing the optogenetic circuit. Engineered *S. cerevisiae* strains (yNCellulose and yNSurface) sense blue light and express the reporter protein NanoLuc in response. The LexA-CRY2 and VP16-CIB synthetic transcription factors are expressed from constitutive promoters *pREV1* and *pTDH3*, respectively. Upon exposure to light, the dimer binds LexA-binding sites (*8xLexA-op*) in the *pLEXA* promoter, activating transcription of the NanoLuc gene. **b**, Schematic illustrating the two modes of functionalization. The yNCellulose strain secretes NanoLuc-CBD that diffuses into and eventually binds the surrounding cellulose matrix, while the yNSurface strain displays NanoLuc-SED1 on the yeast cell surface. **c**, The pellicles grown in the dark or in light after 3 days. **d**, yNCellulose and yNSurface pellicles were grown in the dark and then exposed to light under a mask for 4 h. Pellicles were flipped so that the lower surface, where the majority of yeast cells are localized, was closest to the light source. The NanoLuc substrate was applied at the end for visualization of the pattern created by masking. **e**, yNCellulose and yNSurface pellicles grown in the dark were exposed to a complicated pattern created by a projector. The NanoLuc substrate was applied at the end for visualization of the pattern created by masking.

The functionalized BC materials we generated here could be applied to the degradation of environmental pollutants such as β-lactam antibiotics or oestrogen hormones present in wastewater streams. An advantage of our approach is that secreted enzymes functionalize the material as it forms, so separate steps for protein purification and enzyme–material chemical bonding are not needed in the manufacturing process. This approach is also highly adaptable; numerous other protein targets—enzymes, binding or

adhesion proteins or even structural proteins—could be secreted from *S. cerevisiae* to add the desired properties to BC. Importantly, the feasibility of this will depend on the secretion yield for a given protein, which may be lower under static growth conditions compared to shake-flask or bioreactor growth, as well as the stability of the protein outside the cell or after any required sterilization procedures.

Our approach also offers a fast, simple method to generate biosensor materials. Sense-and-respond ELMs could find application in a variety of contexts, in biosensing, bioremediation or creation of patterned materials. Again, numerous existing *S. cerevisiae* biosensor strains able to detect pathogens[48], environmental pollutants[53] or biomarkers[54] could be plugged into the Syn-SCOBY approach.

Organisms are remarkable material-producing systems, capable of self-assembling complex materials with diverse chemical and physical properties starting only from simple feedstocks. Simultaneously, living cells within these materials control their morphology over multiple length scales and remodel material properties in response to environmental cues. Synthetic material systems capable of recreating all of these behaviours do not exist. As such, the ability to genetically control the process of material self-assembly with the same level of sophistication seen in natural biological materials could revolutionize the manufacture of products for use in numerous arenas of human life and society. The Syn-SCOBY approach showcases the viability of microbial co-cultures combined with synthetic biology tools to design, grow and test functional living materials.

Online content

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Methods

Strains, constructs and DNA assembly. Strains used in this study are listed in Supplementary Table 1. All plasmids used in this study are listed in Supplementary Table 2, with links provided to their complete sequences. All plasmids constructed in this study were constructed using standard cloning techniques. Oligonucleotides were obtained from IDT. Restriction endonucleases, Phusion-HF DNA polymerase and T7 DNA ligase were obtained from NEB. Unless stated, all plasmids were transformed into *Escherichia coli* turbo (NEB) for amplification and verification before transforming into *S. cerevisiae* for protein expression and secretion. Constructs were verified by restriction enzyme digestion and Sanger sequencing (Source Bioscience).

S. cerevisiae constructs and strains were generated using the YTK system developed by the Dueber lab[35]. The YTK system uses Golden Gate assembly to combine preassembled, defined parts into single-gene cassettes and multigene cassettes. The final positions of preassembled parts within constructs are determined by the sequences of 4 bp overhangs created by digestion with type IIS restriction enzymes (BsaI or BsmBI). Users can therefore pick and choose from preassembled promoter, terminator and protein-coding parts to create expression cassettes. The identity of constituent YTK DNA parts used for all single-gene and multigene cassettes are listed in Supplementary Tables 3 and 4, respectively. All parts were cloned into preassembled backbone plasmids containing genetic elements enabling cloning in *E. coli* and later integrative transformation into the *URA3* locus (pYTK096) or the *HO* locus (pWS473) in *S. cerevisiae*. Type 2, 3 and 4 parts were cloned into preassembled backbones. To create more complex fusion proteins, additional subparts were used (for example, 3a and 3b parts). New parts were codon-optimized for *S. cerevisiae* expression, synthesized commercially by GeneArt or IDT and cloned into the YTK system entry vector, pYTK001, for storage and verification. All other parts were taken from the YTK or from published work. Golden Gate assembly reactions were performed as described in Lee et al.[35]

Culture conditions and media. Yeast extract peptone dextrose (YPD) and yeast extract peptone sucrose (YPS) media were prepared with 10 g l^{-1} yeast extract, 20 g l^{-1} peptone from soybean and 20 g l^{-1} glucose or sucrose. Synthetic complete (SC) dropout media were prepared with 1.4 g l^{-1} yeast synthetic dropout medium supplements, 6.8 g l^{-1} yeast nitrogen base without amino acids and 20 g l^{-1} glucose. Depending on the required selection, SC media were supplemented with stock solutions of one or more of uracil (final concentration 2 g l^{-1}), tryptophan (final concentration 50 mg l^{-1}), histidine (final concentration 50 mg l^{-1}) and leucine (final concentration 0.1 g l^{-1}). Hestrin–Schramm (HS) media were prepared with 5 g l^{-1} yeast extract, 5 g l^{-1} peptone from soybean, 2.7 g l^{-1} Na_2HPO_4, 1.5 g l^{-1} citric acid and 20 g l^{-1} glucose or sucrose. Where required, media were supplemented with 20 g l^{-1} bacteriological agar.

E. coli was grown in LB medium at 37 °C, supplemented with appropriate antibiotics at the following concentrations: chloramphenicol, 34 μg ml^{-1} and kanamycin, 50 μg ml^{-1}. For biomass accumulation, *K. rhaeticus* was grown at 30 °C in yeast extract peptone dextrose (YPD) medium supplemented with 34 μg ml^{-1} chloramphenicol and 1% (v/v) cellulase from *T. reesei* (Sigma-Aldrich, C2730). Notably, we found that the growth of *K. rhaeticus* liquid cultures was significantly more reliable when inoculated from glycerol stock, rather than from colonies. Therefore, unless otherwise indicated, all *K. rhaeticus* cultures were inoculated from glycerol stocks. *S. cerevisiae* was grown at 30 °C in rich YPD medium or selective SC medium lacking the appropriate supplements, each supplemented with 50 μg ml^{-1} kanamycin.

Co-culture condition screen. Triplicate samples of *K. rhaeticus* Kr RFP were inoculated from glycerol stocks into 5 ml YPD medium supplemented with cellulase (1% v/v) and grown in shaking conditions for 3 days. Triplicate samples of *S. cerevisiae* yWS167 were inoculated from plates into 5 ml YPD medium and grown in shaking conditions for 24 h. To prepare screens, *K. rhaeticus* and *S. cerevisiae* were inoculated into 2 ml volumes of YPD, YPS, HS-glucose or HS-sucrose media in 24-well cell culture plates. *K. rhaeticus* cultures were diluted 1:50 into fresh media. *S. cerevisiae* cultures were inoculated over a range of dilutions: 1:100, 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000. To enable pellicle formation, plates were incubated for 4 days under static conditions at 30 °C. After 4 days of incubation, cultures were photographed under identical conditions. Where present, pellicle layers were removed from the culture surface and photographed.

OptiPrep concentration screen. Triplicate samples of *K. rhaeticus* Kr were inoculated from glycerol stocks into 5 ml YPD medium supplemented with cellulase (1% v/v) and grown in shaking conditions for 3 days. Triplicate samples of *S. cerevisiae* BY4741 were inoculated from plates into 5 ml YPD medium and grown in shaking conditions for 24 h. To prepare screens, *K. rhaeticus* and *S. cerevisiae* were inoculated into 10 ml volumes of YPS or YPS plus 0, 25, 35, 45, 55 or 65% (w/v) OptiPrep in 10 ml tubes. *K. rhaeticus* cultures were diluted 1:50 into fresh YPS media. *S. cerevisiae* cultures were inoculated at dilution of 1:10,000. To enable pellicle formation, tubes were incubated for 3 days under static conditions at 30 °C. After 3 days of incubation, cultures were photographed under identical conditions.

OptiPrep co-culture condition screen. Triplicate samples of *K. rhaeticus* Kr were inoculated from glycerol stocks into 5 ml YPD medium supplemented with cellulase (1% v/v) and grown in shaking conditions for 3 days. Triplicate samples of *S. cerevisiae* BY4741 were inoculated from plates into 5 ml YPD medium and grown in shaking conditions for 24 h. To prepare screens, *K. rhaeticus* and *S. cerevisiae* were inoculated into 10 ml volumes of YPS or YPS plus 40% (w/v) OptiPrep in 50 ml tubes with breathable caps. *K. rhaeticus* cultures were diluted 1:50 into fresh YPS media. *S. cerevisiae* cultures were inoculated over a range of dilutions: final OD_{600} = 1:100, 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000. To enable pellicle formation, tubes were incubated for 3 days under static conditions at 30 °C. After 3 days of incubation, cultures were photographed under identical conditions. Pellicles were collected and washed/shaken in deionized water at 4 °C for 12 h, twice. Clean pellicles were subjected to lyophilization for 3 days and measured for dry weight. To remove *S. cerevisiae* and *K. rhaeticus* cells associated with the cellulose matrix, pellicles were immersed in 0.1 M NaOH at 65 °C for 4 h. Washed pellicles were again washed/shaken in H_2O at 4 °C for 6 h, subjected to lyophilization for 3 days and measured for dry weight.

Testing of different supplements. Starter cultures of *K. rhaeticus* were grown in 2X HS-Media (supplemented with 2% (v/v) cellulases) at 30 °C and 250 r.p.m. for 3 days. The OD_{700} was adjusted to 2.5 and the co-culture inoculated with a 1:50 dilution of the adjusted starter culture. *S. cerevisiae* starter culture was grown overnight at 30 °C and 250 r.p.m. in YPS media. The OD_{700} of yeast was adjusted to 0.01 and the co-culture was inoculated with 1:100 dilution.

In order to screen different supplements, the co-culture media was composed of 5X YPS (adjusted to a final concentration of 1X), autoclaved water and the different supplements (poly(ethylene glycol) with a molecular mass of 3,000, 8,000 or 20,000 Da to a final concentration of 100, 80 or 60 mg ml^{-1}, poly(ethylene glycol) with a molecular mass of 900,000 Da to a final concentration of 40, 30 or 20 mg ml^{-1}, alginate to a final concentration of 20, 15 or 10 mg ml^{-1} and iodixanol (OptiPrep) to a final concentration of 48, 40, 32, 24 or 16% (v/v)).

After mixing the components, the media was aliquoted in 12-well plates with 3 ml in each well and inoculated with a master mix of the starter co-culture already diluted. The static co-culture was incubated at 30 °C for 3 days.

Pellicles, if formed, were removed from the top of the static co-culture and dried by pressing them between adsorbent paper for 1 day. Dried pellicles were imaged with a fluorescence laser scanner (FLA-5000, Fujifilm). This was possible as the yeast cells were expressing mScarlet-I, a red fluorescent protein (yWO68). After imaging, the pellicles were digested by incubating individual pellicles in 2 ml of 1X PBS buffer supplemented with 5% (v/v) cellulases at room temperature overnight. Next, the digestion mix was centrifuged (6,500 *g* for 5 min) and the supernatant removed. The pellet was formed solely by the biomass of yeast and *K. rhaeticus*. The cell pellet was resuspended in 400 μl 1X PBS and pipetted into two wells of a 96-well plate. The red fluorescence was determined with a spectrophotometer (BioTek Synergy HT) and used as the overall yeast incorporation value.

Standard co-culture protocol. Unless otherwise stated, all co-cultures were prepared using *K. rhaeticus* Kr RFP and *S. cerevisiae* yWS167 strains, allowing facile strain-specific detection through fluorescence measurements. Triplicate samples of *K. rhaeticus* were inoculated from glycerol stocks into 5 ml YPD medium supplemented with cellulase (1% v/v) and grown in shaking conditions for 3 days. Triplicate samples of *S. cerevisiae* were inoculated from plates into 5 ml YPD medium and grown in shaking conditions for 24 h. To enable inoculation of co-cultures with equivalent cell densities of different samples, OD_{600} measurements were made and used to normalize pre-culture densities. *K. rhaeticus* pre-cultures were centrifuged at 3,220 *g* for 10 min and cell pellets resuspended in a sufficient volume of YPS medium to result in a final OD_{600} of 2.5. *S. cerevisiae* pre-cultures were diluted in YPS medium to a final OD_{600} of 0.01. To prepare final co-cultures, resuspended *K. rhaeticus* samples were diluted 1:50 and pre-diluted *S. cerevisiae* samples were diluted 1:100 into fresh YPS medium. In instances where strains were inoculated into various different final media, *K. rhaeticus* pellets were resuspended in PBS buffer and *S. cerevisiae* cultures were pre-diluted in PBS buffer. To prepare OptiPrep-containing co-cultures, OptiPrep (D1556, Sigma-Aldrich) was added to YPS media to a final concentration of 45% (v/v). Co-cultures were grown in either 55 mm Petri dishes (15 ml) or 12-well cell culture plates (4 ml). Co-cultures were incubated for 3 d at 30 °C under static conditions. It is important to ensure that culture vessels are not mechanically disturbed during the incubation period as this can partially submerge the growing BC layer, resulting in the formation of multiple, disconnected BC layers.

Determining BC pellicle yields. To determine the yields of BC pellicles, pellicle layers were removed from the surfaces of cultures and dried using the 'sandwich method'. Here, pellicles were sandwiched between sheets of grease-proof paper and then further sandwiched between multiple sheets of absorbent paper and finally placed under a heavy weighted object. After 24 h, fresh sheets of absorbent paper were added and pellicles left for an additional 24 h. Pellicles dried in this way were

then weighed to determine pellicle yields. Importantly, pellicles were not treated with NaOH to lyse and remove cells embedded within the BC matrix. Since the cellular biomass constitutes an integral, functional part of BC-based ELMs, we chose not to perform NaOH washes.

This method was used to follow the yields of pellicle formation over time. Here, multiple co-cultures were prepared in triplicate using the standard co-culture procedure. Co-cultures were grown in 12-well plate format. At specific time points, pellicle layers were removed to be dried and weighed.

Co-culture passage. To test whether co-cultures could be passaged, initial co-cultures were prepared in triplicate in 15 ml YPS cultures using the standard co-culture protocol. After incubation for 3 d at 30 °C, photographs were taken of the resultant cultures. To initiate new rounds of growth, pellicle layers were removed and the liquid below mixed by aspiration then diluted 1:100 into fresh samples of 15 ml YPS. This process was repeated over 16 rounds.

To confirm that the initial strain of GFP-expressing *S. cerevisiae* (yWS167) was maintained during passage, samples were plated at the end of each round. Samples from both the liquid below the pellicle and the pellicle layer itself were plated at various dilutions onto YPD-kanamycin plates. To enable plating, pellicles were first digested by shaking gently for 16 h at 4 °C in 15 ml of PBS buffer with 2% (v/v) cellulase from *T. reesei* (Sigma Aldrich, C2730). After 48 h of incubation at 30 °C, plates were imaged for GFP fluorescence. Dilutions were selected that enabled visualization of single colonies. Initially, plates were imaged using a Fujifilm FLA-5000 Fluorescent Image Analyser. However, due to equipment malfunction, later plates were photographed under a transluminator.

Determining cell distribution in co-cultures. Cell distributions were determined by plating samples of cells onto solid media and counting the resultant colonies. Pellicle samples were first gently rinsed by inverting ten times in 15 ml PBS and then digested by shaking gently for 16 h at 4 °C in 15 ml of PBS buffer with 2% (v/v) cellulase from *T. reesei* (Sigma Aldrich, C2730). Samples were diluted at various levels into PBS. For *S. cerevisiae* cell counts, samples were plated onto YPD-kanamycin media. For *K. rhaeticus* cell counts, samples were plated onto SC media lacking all four supplements essential for *S. cerevisiae* yWS167 growth (histidine, leucine, tryptophan and uracil). In all instances, Kr RFP and yWS167 strains were used. Despite the use of selective growth conditions, to ensure the colonies counted were the target strains, plates were scanned for fluorescence using a Fujifilm FLA-5000 Fluorescent Image Analyser. Plate cell counts were used to calculate the original colony forming units (c.f.u.) per unit volume for liquid samples. However, since the exact volumes of pellicle were not measured prior to degradation, it was not possible to calculate the exact cell counts in c.f.u. per unit volume. To enable a rough approximation of the cell counts per unit volume, pellicle volumes were estimated at fixed levels and these values were used to calculate estimated c.f.u. per unit volume. For 15 ml cultures, pellicle volumes were estimated at 4 ml, and for 4 ml cultures in 12-well plates, pellicle volumes were estimated at 1 ml.

To compare cell counts from monocultures and co-cultures of *K. rhaeticus* and *S. cerevisiae*, pre-cultures of *K. rhaeticus* Kr RFP were pelleted and resuspended in PBS buffer and pre-cultures of *S. cerevisiae* yWS167 were diluted in PBS buffer, according to the standard co-culture procedure. Various co-cultures and monocultures were then prepared in different media in 15 ml volumes. After incubation for 3 d at 30 °C, pellicle and liquid samples were prepared, diluted and plated for cell counts.

To determine the reproducibility of co-culture cell counts, co-cultures were prepared according to the standard co-culture protocol in 15 ml cultures on three separate occasions. After incubation for 3 d at 30 °C, pellicle and liquid samples were prepared, diluted and plated for cell counts.

Invertase supplementation experiment. Co-cultures and *K. rhaeticus* Kr RFP monocultures were prepared in YPS medium according to the standard co-culture procedure. Recombinant, purified *S. cerevisiae* invertase (Sigma-Aldrich, I9274) was resuspended in 100 mM citrate buffer (pH 4.5) to create a stock solution at a final concentration of 5 U μl^{-1}. This stock solution was diluted into YPS medium for a range of final invertase concentrations (50 mU ml^{-1} (10^{-2}), 5 mU ml^{-1} (10^{-3}), 0.5 mU ml^{-1} (10^{-4}), 50 μU ml^{-1} (10^{-5})). After 3 days of growth at 30 °C, cultures (and, where present, pellicles) were imaged.

Supernatant nitrocefin assay. For culture supernatant assays, WT BY4741, yCG04 and yCG05 *S. cerevisiae* strains were grown in triplicate overnight in YPD liquid medium with shaking. After 16 hours of growth, liquid cultures were back-diluted to a final OD_{600} of 0.01 in 5 ml fresh YPS medium and grown for 24 h with shaking. The resultant cultures were centrifuged at 3,220 *g* for 10 min, and the supernatant fractions were harvested. Supernatant samples were pipetted in 50 µl volumes into the wells of a 96-well plate. The colorimetric substrate, nitrocefin (484400, Merck-Millipore), was resuspended in dimethylsulfoxide to create a 10 mg ml^{-1} working stock. This stock was diluted to 50 µg ml^{-1} in nitrocefin assay buffer (50 mM sodium phosphate, 1 mM EDTA, pH 7.4). To start the reaction, 50 µl of nitrocefin at 50 µg ml^{-1} was added to each of the samples simultaneously, and the absorbance at 490 nm was measured over time. Active BLA converts nitrocefin to a red substrate, increasing the absorbance of light at 490 nm. Therefore, to calculate the relative BLA activity in samples, the rate of change in the absorbance of light at 490 nm was determined. Specifically, the product formation rates were calculated from the gradient over the linear region of a graph plotting fluorescence against time.

Pellicle nitrocefin assays. For initial pellicle assays (Fig. 2e,f), WT BY4741, yCG04 and yCG05 *S. cerevisiae* strains were co-cultured with *K. rhaeticus* (Kr RFP) in triplicate, according to the standard co-culture protocol. Following 3 days of growth, pellicles were removed and washed in 15 ml PBS buffer for 30 min with shaking at 150 r.p.m. Square pieces of pellicle, measuring 5 mm × 5 mm, were then cut using a scalpel. The remainder of the pellicle was dried using the sandwich method. Once dried, pellicles were again cut to produce 5 mm × 5 mm pieces. Dried pellicle pieces were rehydrated by adding 25 µl of PBS buffer and incubating for 30 min. Assays for both wet and dried samples were run by adding 10 µl of nitrocefin, diluted to 1 mg ml^{-1} in PBS buffer, to each of the pellicle pieces simultaneously. Initial assays were performed at room temperature. Photographs were taken of pellicles over the course of 35 min to follow the colour change. To provide a quantitative measure of colour change, the ImageJ (NIH) image analysis software was used. Images were first split into individual colour channels. Since yellow-to-red colour change is caused by an increase in the absorbance of green light wavelengths, the green channel was selected. To quantify the yellow-to-red colour change, the green channel intensity was then measured from greyscale-inverted images of pellicle slices over time. Since preliminary results showed that WT pellicles exhibited no colour change, the signal from WT pellicles was used as a baseline value to correct for background levels of green channel intensity.

To determine absolute levels of BLA activity in wet and dried pellicles, a similar protocol was used to create standard curves. Standard curves were prepared using a commercial *E. coli* BLA enzyme (ENZ-351, ProSpec). First, pellicles grown with WT BY4741 *S. cerevisiae* were washed in nitrocefin assay buffer (50 mM sodium phosphate, 1 mM EDTA, pH 7.4). Pellicle pieces measuring 5 mm × 5 mm were cut and weighed to enable determination of the approximate volume of liquid within the pellicle. The remainder of the pellicle was dried using the sandwich method. Once dried, 5 mm × 5 mm pieces of pellicle were cut for dried pellicle standard curves. Dried pellicle pieces were rehydrated by adding 20 µl of nitrocefin assay buffer. Pre-diluted standard BLA samples were then added to pellicle pieces in 5 µl volumes and allowed to diffuse throughout the BC for 30 min. To initiate the reaction, 5 µl aliquots of nitrocefin, diluted to 2 mg ml^{-1} in nitrocefin assay buffer, were added to each of the pellicle pieces simultaneously. Samples were incubated at 25 °C and photographs taken over the course of the reaction. Again, ImageJ was used to quantify the yellow-to-red colour change at given time points. Time points were chosen to maximize the dynamic range without reaching saturation. For wet pellicles, it was necessary to use the measured weight of pellicle slices to determine the actual final concentration of the standard BLA. Standard curves are shown in Supplementary Fig. 12. Standard curves using fresh wet pellicles, dried pellicles and dried pellicles stored for 1 month or 6 months at room temperature were all prepared according to this method. For long-term storage, dried pellicles were stored in Petri dishes at room temperature and protected from light.

Alongside standard curves, pellicles grown with yCG05 *S. cerevisiae* were analysed using an identical protocol. To enable cross-comparison with standard curves, negative samples (pellicles from co-cultures with WT *S. cerevisiae*) and positive samples (pellicles from co-cultures with WT *S. cerevisiae* to which a known amount of BLA standard had been added) were run with samples. For samples to which no standard BLA was added, 5 µl of nitrocefin assay buffer was added to maintain equal final liquid volumes. Photographs taken at identical time points were then used with standard curves to calculate absolute values of BLA activity. Again, ImageJ was used to quantify the yellow-to-red colour change. For wet pellicles, it was necessary to use the measured weight of pellicle slices to determine the actual final concentration of enzyme. Again, fresh wet pellicles, dried pellicles and dried pellicles stored for 1 month at room temperature were all assayed according to this method.

BLA activity retention assay. To determine the retention of BLA within BC following multiple rounds of washes, nitrocefin assays were performed. Pieces measuring 5 mm × 5 mm were cut from dried pellicles grown with yCG04 and yCG05. All pellicle pieces were rehydrated by incubating in 1 ml of PBS buffer. Pieces were subjected to a variable number of wash steps, where pellicle pieces were incubated in 4 ml PBS buffer at 25 °C and 150 r.p.m. for 30 min. After washing, pellicles were assayed for BLA activity. Negative samples (pellicles from co-cultures with WT *S. cerevisiae*) and positive samples (pellicles from co-cultures with WT *S. cerevisiae* to which a known amount of BLA standard had been added) were run alongside all samples. For samples to which no standard BLA was added, 5 µl of PBS buffer was added to maintain equal final liquid volumes. As before, assays were initiated by adding 5 µl of nitrocefin, diluted to 2 mg ml^{-1} in PBS buffer, to each of the pellicle pieces simultaneously. Samples were run in batches based on the number of washes. Again, ImageJ was used to quantify the yellow-to-red colour change at given time points. To enable cross-comparison between different assay runs, negative samples were used to subtract background signals and positive

samples were used to normalize signals. To ensure that yellow-to-red colour change values were within a range in which there is a linear relationship between BLA activity and the yellow-to-red colour change signal, a standard curve was run. The standard curve ($r^2 = 0.9571$) confirmed that detected yellow-to-red colour change values fell within the linear range.

X-α-Gal α-galactosidase assays. A stock solution of X-α-Gal (Sigma-Aldrich, 16555) was prepared in dimethylsulfoxide at a concentration of 40 mg ml^{-1}. For plate assays, 100 µl of X-α-Gal was spread on plates prior to cell plating and images were taken after 3 days of growth at 30 °C. For pellicle assays, pellicles grown with *K. rhaeticus* Kr RFP were harvested after 3 days of growth, following the standard co-culture procedure. Pellicles were then washed in 15 ml 100 mM citrate buffer (pH 4.5) for 30 min with shaking at 150 r.p.m. Square pieces of pellicle, measuring 5 mm × 5 mm, were then cut using a scalpel. The remainder of the pellicle was dried using the sandwich method. Once dried, pellicles were again cut to produce 5 mm × 5 mm pieces. Dried pellicle pieces were rehydrated by adding 25 µl of 100 mM citrate buffer, pH 4.5 and incubating for 30 min. Assays for both wet and dried samples were run by adding 2.5 µl of X-α-Gal stock solution and incubating at 25 °C. Images were taken over the course of several hours.

ABTS laccase activity assays. Stock solutions were prepared of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma-Aldrich, A1888) at a final concentration of 0.1 M and copper sulphate at a final concentration of 1 M. Laccases are copper-containing enzymes, requiring supplementation of copper for culture and assay conditions. For plate assays, 125 µl of 0.1 M ABTS and 25 µl of 1 M $CuSO_4$ were spread on plates prior to cell plating and images taken after 3 days of growth at 30 °C. For pellicle assays, pellicles grown with *K. rhaeticus* Kr RFP were harvested after 3 days of growth following the standard co-culture procedure. The only modification was the addition of 1 mM $CuSO_4$ to the culture medium of both *S. cerevisiae* pre-cultures and co-cultures. Pellicles were then washed in 15 ml 100 mM citrate buffer, 1 mM $CuSO_4$, pH 4.5 for 30 min with shaking at 150 r.p.m. Square pieces of pellicle, measuring 5 mm × 5 mm, were then cut using a scalpel. The remainder of the pellicle was dried using the sandwich method. Once dried, pellicles were again cut to produce 5 mm × 5 mm pieces. Dried pellicle pieces were rehydrated by adding 25 µl of 100 mM citrate buffer, 1 mM $CuSO_4$, pH 4.5 and incubating for 30 min. Assays for both wet and dried samples were run by adding 5 µl of ABTS stock solution and incubating at 25 °C. Images were taken over the course of several hours.

Scanning electron microscopy (SEM). Pellicles were grown for 3 days following the co-culture procedure and washed with deionized water three times (shaking at 70 r.p.m. at 4 °C for 12 h per wash) to remove residual YPS or OptiPrep. Washed pellicles were then freeze-dried with a lyophilizer for at least 48 h before coated with a gold sputter. Images were taken with a JEOL 6010LA benchtop scanning electron microscope.

Environmental SEM (eSEM). Pellicles were grown and washed as described in the SEM sample preparation. Instead of being subjected to freeze-drying, washed pellicles were placed in 6-well plates suspended in transfer buffer containing 2.5% glutaraldehyde (10% EM grade from Electron Microscopy Sciences, 16100) and 200 mM sodium cacodylate buffer at pH 7.2 (Electron Microscopy Sciences, 11655). After 60 min of fixation, samples were rinsed twice with the cacodylate buffer for 5–10 minutes each at 4 °C, followed by being rinsed three or four times with distilled water. Rinsed samples should then be subjected to dehydration as soon as possible; thus, they were serially dehydrated with multiple rounds of ethanol (5 min each of 35, 45, 55, 65, 70, 85, 95 and 100% ethanol). After dehydration, samples were transferred to 50% tetramethylsilane (Electron Microscopy Sciences, 21760) mixed with 50% ethanol and incubated for 15 min, followed by transferring to 80% tetramethylsilane mixed with 20% ethanol and another 15 min incubation. Finally, samples were transferred to 100% tetramethylsilane and incubated for 5 minutes, repeated three times and air-dried overnight in the fume-hood. Images were taken with a FEI XL30 eSEM used on low vacuum mode with a back-scattered electron detector. Dehydrated samples were placed on the stub using double-sided conductive carbon tape (Electron Microscopy Sciences). Parameters were set as follows: working height <10 cm, low pressure setting >2.5 torr, accelerating voltage 15 kV, magnification greater than ×1,000 and spot size of 3.

Cell leakage from pellicles. Pellicles were grown in YPS on a 12-well plate for 3 days to reach ~900 mg in wet weight and then subjected to three rounds of washing in 20 ml PBS (shaking at 70 r.p.m. at 4 °C for 24 h per wash). Total c.f.u. counts per pellicle were obtained from cellulase-digested pre-wash pellicles, liquid (PBS) after each wash step and post-wash pellicles.

Brunauer–Emmett–Teller (BET) surface area analysis. Freeze-dried pellicles were cut into 5 mm × 5 mm pieces and placed in sample tubes for 1 h to degas at 423 K using a Micromeritics ASAP 2020 analyser. BET surface area and pore size were then determined with N_2 adsorption at 77 K using BET and Barrett–Joyner–Halenda analyses on the same instrument.

Preparing and assaying sense-and-response pellicles. In yGPH093, transcription from the BED-inducible promoter is controlled by a synthetic transcription factor (Z_3EV) consisting of three domains: the Zif268 DNA-binding domain, the human oestrogen receptor (hER) ligand-binding domain and the transcriptional activation domain of viral protein 16 ($VP16^{AD}$)[45]. When present, BED binds to the hER ligand-binding domain of Z_3EV, releasing it from its basal sequestration in the cytosol and enabling it to translocate into the nucleus. Once in the nucleus, the Zif268 domain binds cognate DNA sequences in engineered promoters, and the $VP16^{AD}$ domain activates transcription of downstream genes. As a preliminary test of *S. cerevisiae* sense and response in BC pellicles, co-cultures were prepared in triplicate according to the standard co-culture protocol using WT BY4741 and yGPH093 strains. Co-cultures were inoculated into 4 ml YPS+OptiPrep medium in 12-well cell culture plates. After 3 days of growth, pellicles were removed and washed by incubating at 25 °C with shaking at 150 r.p.m. in 15 ml PBS. Pellicles were then placed in 15 ml of fresh YPD medium in the presence or absence of 5 nM BED (E8875, Sigma-Aldrich) and incubated for 24 h at 30 °C and 150 r.p.m. During growth, cells had 'escaped' from biosensor pellicles, making the medium surrounding the pellicles turbid. Therefore, to remove loosely associated cells, pellicles were washed twice by incubating for 30 min at 25 °C and 150 r.p.m. in 15 ml of PBS buffer. Finally, pellicles were imaged simultaneously for GFP fluorescence under a transilluminator.

Similarly, dried biosensor pellicles were prepared in triplicate according to the standard co-culture protocol using WT BY4741 and yGPH093 or WT BY4741 and yWS890 strains. Co-cultures were inoculated into 4 ml YPS+OptiPrep medium in 12-well cell culture plates. After 3 days of growth, pellicles were dried using the 'sandwich method'. Dried pellicles were then placed in 15 ml of fresh YPD medium in the presence or absence of 5 nM BED or 50 nM *S. cerevisiae* α-mating factor (RP01002, Genscript) and incubated for 24 h at 30 °C. Notably, to more closely match the potential use of biosensors in an on-site detection setting, pellicles were incubated without agitation in this and all future experiments. Since static growth results in far less growth in the surrounding liquid, pellicles were only briefly washed after incubation by inverting ten times in 15 ml PBS buffer. Finally, pellicles were imaged side by side for GFP fluorescence under a transilluminator. To test for stability after long-term storage, pellicles were stored for 4 months at room temperature stored in Petri dishes protected from light. These pellicles were cut in half prior to induction, which was performed as described above.

The BED-inducible CtLcc1-secreting strain yCG23 was initially screened for laccase induction using a plate-based ABTS assay. Transformants of yCG23 were re-streaked in triplicate on SC URA$^-$ plates supplemented with 125 µl of 0.1 M ABTS and 25 µl of 1 M $CuSO_4$. After incubation for 3 d at 30 °C, colonies were imaged. Co-cultures between *K. rhaeticus* Kr RFP and yCG01 or yCG23 were then prepared in triplicate in 12-well plate format, using YPS+OptiPrep medium supplemented with 1 mM $CuSO_4$. After 3 days of growth, pellicles were harvested and were washed by incubating for 30 min at 25 °C and 150 r.p.m. in 15 ml of 100 mM citrate buffer, 1 mM $CuSO_4$, pH 4.5. Pellicles were then inoculated into 15 ml of fresh YPD supplemented with 1 mM $CuSO_4$ in the presence or absence of 5 nM BED and statically incubated at 30 °C for 24 h. After incubation, pellicles were washed by incubating for 30 min at 25 °C and 150 r.p.m. in 15 ml of 100 mM citrate buffer, 1 mM $CuSO_4$, pH 4.5. Pellicles were then placed in a 12-well plate and 75 µl of 0.1 M ABTS was added to each well to assay for laccase activity. Pellicles were incubated at 25 °C and imaged after 72 h.

Determining the viability of *S. cerevisiae* in dried BC pellicles. Co-cultures were prepared in triplicate according to the standard co-culture protocol. Co-cultures were inoculated into 4 ml YPS+OptiPrep medium in 12-well cell culture plates. Counts of viable *S. cerevisiae* cells within wet and dried pellicles were determined as described in Determining cell distribution in co-cultures. Dried pellicles were also stored for 1 month at room temperature and then degraded and plated onto YPD medium. Since one of the triplicate samples produced no colonies, we could not calculate estimated cell counts within pellicles. However, images are presented of the three plates to show that viable cells were indeed recovered from the other two samples (Supplementary Fig. 28d).

Total cellulase activity assay. *S. cerevisiae* strains BY4741 and yCelMix were grown overnight in YPS in triplicate with shaking. After 16 h of growth, liquid cultures were back-diluted to a final OD_{600} of 0.1 in 5 ml fresh YPS medium with 2 mM L-ascorbic acid (A7506, Sigma-Aldrich) and grown for 24 h with shaking. The resultant cultures were centrifuged at 3,220 *g* for 10 min and the supernatant fractions harvested. Supernatant samples were pipetted in 50 µl volumes into the wells of a 96-well plate. The EnzChek Cellulase Substrate (E33953, Thermo-Fisher) was resuspended in 50% dimethylsulfoxide and diluted fivefold in 100 mM sodium acetate (pH 5.0). To start the reaction, 50 µl of cellulase substrate was added to the supernatant and let incubate for 30 min in the dark at room temperature. To build an enzyme activity standard curve, the cellulase from *T. reesei* (C2730, Sigma-Aldrich) was used to prepare a serial dilution in YPS medium and mixed with the substrate at 1:1 ratio. Blue fluorescence (360 or 460 nm) was detected using a plate reader (Synergy H1, BioTek) after 30 min of incubation in the dark at room temperature. The data from the enzyme standards were fit to an exponential model, $a\exp(bx) + c\exp(dx)$ in MATLAB. This model was then used to calculate

the total cellulase activity of the supernatant from yCelMix (using supernatant from BY4741 as a blank control).

Pellicle tensile test. Co-cultures were set up in 40 ml YPS+OptiPrep (plus 2 mM L-ascorbic acid) and grown in square plates (100 mm × 15 mm) for 2 d at 30 °C. Pellicles were then washed in deionized water three times (shaking at 70 r.p.m. at 4 °C for 12 h per wash) and dried using the sandwich method described previously but with an extended 3 d drying time to ensure water removal. Dried pellicles were cut into 60 mm × 10 mm strips and their thicknesses were measured with a micrometer. Tensile test was performed with a Zwick mechanical tester (BTC-ExMacro .001, Roell) following the ASTM D882 protocol at 1 mm min^{-1} speed.

Pellicle rheology analysis. The rheological properties of washed pellicles were characterized on a rheometer (AR2000, TA Instruments) with a 25 mm environmental test chamber (ETC) aluminium plate (1 mm gap). The strain sweep measurements were taken from 0.01% to 100% strain amplitude at a constant frequency of 1 rad s^{-1} while frequency sweep measurements were taken from 0.1 rad s^{-1} to 100 rad s^{-1} at a constant strain amplitude of 1%. Samples were kept fully hydrated with deionized water at 25 °C on a Peltier thermoelectric plate.

Light-inducible circuit promoter characterization. Yeast strains were grown overnight in YPD in triplicate with shaking. After 16 hours of growth, liquid cultures were back-diluted to a final OD_{600} of 0.2 in 100 µl fresh YPD and pipetted into the wells of two 96-well plates (duplicates). One of the two plates was wrapped in black aluminium foil as a dark control. Both plates were placed under an LED lamp at 30 °C for 4 h. Green fluorescence was then measured with a plate reader.

Light-inducible luciferase assay. Yeast strains were grown overnight in YPD in triplicate with shaking. After 16 hours of growth, liquid cultures were back-diluted to a final OD_{600} of 0.2 in 15 µl fresh YPS and pipetted into the wells of two 96-well plates (duplicates for light and dark conditions, as previously described). Plates were placed under an LED lamp at 30 °C for 4 h. Substrates in buffer from the Nano-Glo Luciferase Assay System (N1120, Promega) were added to the culture at a 1:1 ratio at the end of the incubation. After incubation in the dark for 5 min, the bioluminescence of the samples was measured with a plate reader.

Light-inducible pellicle response assay. Co-cultures were set up using yeast strains BY4741, yNCellulose and yNSurface along with WT *K. rhaeticus* in 10 ml YPS+OptiPrep. For the long-term exposure experiments, 60 mm Petri dishes were prepared as duplicates; one was wrapped in black aluminium foil, the other was not. The plates were placed under an LED lamp at 30 °C for 3 d. After the incubation, pellicles were flipped so the bottom side was facing up and transferred onto YPD agar plates. Nano-Glo mix (500 µl) was applied to the pellicles evenly over the entire surface. After incubation in the dark for 10 min, bioluminescence of the samples was detected with a ChemiDoc Touch imager (BioRad). For the short-term exposure experiments (masking), co-cultures were grown in the dark at 30 °C for 3 d. Pellicles were flipped so the bottom side was facing up and transferred onto YPD agar plates. A mask made of black aluminium foil with a carved pattern in the centre was placed on top of the pellicles. Plates were placed under an LED lamp and incubated at 30 °C for 4 h. The mask was then removed, and 500 µl of Nano-Glo mix was applied to the pellicles evenly over the entire surface. After incubation in the dark for 10 min, the bioluminescence of the samples was detected with a ChemiDoc Touch imager.

Light patterning on pellicles. Co-cultures were grown in 100 mm square plates protected from light, as previously described. Pellicles were rinsed in PBS, flipped, placed on YPD agar and placed in an incubator with a projector mounted on top. After incubation under the projected pattern (with no lid so as to prevent water condensation) at 30 °C for 12 hours or more, 3 ml of Nano-Glo mix was applied to the pellicles evenly over the entire surface. Bioluminescence images were detected with a ChemiDoc Touch imager after 30 min incubation in the dark.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All produced data that support the main figures of this study are included in this published article. Data points for the mechanical and rheological tests are provided as Source data files. Additional data are available from the corresponding author upon request.

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Author contributions

C.G., T.-C.T. and T.E. conceived and designed the experiments. C.G., T.-C.T. and W.O. performed the Syn-SCOBY co-culture characterization experiments. C.G. performed the BC functionalization and biosensor experiments. T.-C.T. performed yeast incorporation, BC material property modification and optical-patterning experiments. B.A.D. generated yeast strains for optical patterning. W.M.S. generated yeast biosensor strains and genetic tools. G.L.S. performed the eSEM experiments. T.K.L. and T.E. supervised the project and C.G., T.-C.T., W.O. and T.E. wrote the manuscript.

Competing interests

C.G., T.-C.T., W.O., T.K.L. and T.E. are co-inventors on patent applications (International Patent Application no. PCT/US2020/047330) filed by MIT and Imperial College London relating to all the work covered in this article.

Additional information

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Scanning electron microscopy images were collected using a JEOL 6010LA benchtop scanning electron microscope with InTouch Scope V3.01. Brunauer-Emmett-Teller (BET) surface area analysis data were collected using a Micromeritics ASAP 2020 analyzer with ASAP 2020 V3.03. Rheological analysis data were collected using an AR2000 rheometer (TA Instruments) with Rheology Advantage Instrument Control AR v5.7.1 and Rheology Advantage Data Analysis V5.7.0. Tensile test data were collected using a Zwick mechanical tester (BTC-ExMacro .001, Roell) with testXpert III V1.11. Fluorescence and absorbance data were collected using a plate reader (Synergy H1, BioTek) with Gen5 V1.11.5.
Data analysis	Excel and Prism were used to compile and analyze data. Microsoft Excel for Mac V16.16.17. GraphPad Prism V8.4.3. ImageJ for Windows 1.8.0_172

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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All produced data that support the main figures of this study are included in this published article. Data points for the mechanical and rheological tests are provided as source data files. Additional data are available from the corresponding authors upon request.

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Sample size	No sample size calculation was performed. Materials were synthesized from at least three different batches.
Data exclusions	No data were excluded from the analyses.
Replication	All measurements were replicated successfully at least three times. Biological triplicates were performed independently. Material characterization was performed at the same time.
Randomization	No random sample allocation methods were used, as randomization is not relevant for the material characterization performed in this study.
Blinding	Blinding was not applied to the performed studies, as no participants groups were involved.

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Methods

n/a	Involved in the study
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