nature reviews bioengineering

https://doi.org/10.1038/s44222-023-00110-z

Perspective

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Genetically targeted chemical assembly

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Abstract

Cell type-specific interfaces within living animals will be invaluable for achieving communication with identifiable cells over the long term, enabling applications across many scientific and medical fields. However, biological tissues exhibit complex and dynamic organization properties that pose serious challenges for chronic cell-specific interfacing. A new technology, combining chemistry and molecular biology, has emerged to address this challenge: genetically targeted chemical assembly (GTCA), in which specific cells are genetically programmed (even in wild-type or non-transgenic animals, including mammals) to chemically construct non-biological structures. Here, we discuss recent progress in genetically targeted construction of materials and outline opportunities that may expand the GTCA toolbox, including specific chemical processes involving novel monomers, catalysts and reaction regimes both de cellula (from the cell) and ad cellula (towards the cell); different GTCA-compatible reaction conditions with a focus on light-based patterning; and potential applications of GTCA in research and clinical settings.

Sections

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Key points

- Genetically targeted chemical assembly (GTCA) uses cell-specific genetic information to guide the assembly of functional materials in situ.
- The GTCA toolbox can be expanded through specific chemical processes involving novel monomers, catalysts and reaction conditions or regimes.
- GTCA allows both building structures from the targeted cell membrane (de cellula) and an alternative approach (ad cellula) for cell-specific attachment of partially synthesized materials.
- Different GTCA-compatible reaction conditions can be imposed through modulation of light, pH, heat and other signals.
- The broad GTCA concept can be applied for both fundamental research and the treatment of diseases in the central and peripheral nervous systems.

Introduction

The intricate and dynamic physical architecture of biology, particularly in nervous systems, poses substantial challenges to establishing cell type-specific and minimally invasive connections with external interfaces. The human brain, for example, contains tens of billions of intertwined neurons, each using electrical information-propagation signals on the millisecond timescale but with a vast diversity of functional subtypes. Relative to these natural properties, existing hardware for studying the brain lacks sufficient spatiotemporal resolution and specificity. Owing to these mismatches with biological elements and the inability of hardware to target specific cell types[1–10], modern devices cannot yet achieve the goal of high-content, specific, seamless and minimally invasive integration.

A fundamentally new approach to address this mismatch is to genetically endow specific cells within living tissue (for instance, neurons in the brain) with the ability to incorporate materials and build structures with desired forms and functions. Although it has long been possible to modify and regulate biopolymer synthesis from natural building blocks[11–13], these approaches have primarily focused on single-celled microorganisms (thus not addressing the need for cell specificity in multicellular animals); moreover, the diversity of natural substrates remains limited compared with non-natural building blocks that could, theoretically, be recruited for new domains of materials synthesis and assembly, all within living systems. Previous work has shown that conductive polymers can be directly synthesized in living brains without compromising brain function[14]; however, these approaches still cannot target specific cell types.

We have taken the first step towards genetically targeted synthesis using non-biological reactions and reactants to establish the field of genetically targeted chemical assembly (GTCA)[15,16]. The first example of GTCA used cell-specific genetic information to guide neurons to initiate deposition of polymer materials in situ with different electrical conduction properties[15,16]. In this Perspective, we first lay out a road map of strategies to broaden the scope of GTCA by highlighting reported methods alongside new approaches for the chemical synthesis of materials in living systems (including an alternative ad cellula approach for attachment of pre-synthesized materials to cells). Second, we review different GTCA reaction conditions that may be imposed through modulation of light, pH, heat and other signals. Last, we discuss potential applications of the broad GTCA concept in neuroscience research and the treatment of disease, both in the central and peripheral nervous systems, noting existing challenges and future opportunities.

GTCA of functional materials

The first demonstration of GTCA used cell-specific genetic information to guide neurons to deposit conductive or insulating polymers in situ[15]. Specifically, neurons (including in non-transgenic mammals) were genetically engineered to express an enzymatic peroxidase, which catalyses hydrogen peroxide (H_2O_2)-enabled oxidative polymerization. In vitro and in vivo syntheses of conductive polymers such as polyaniline (PANI) and insulating polymers such as poly(3,3′-diaminobenzidine) (PDAB) were demonstrated, and electrophysiological and behavioural studies confirmed that deposited polymers modulated membrane capacitance and cell type-specific behaviours in living neural systems and behaving animals.

Despite this initial success, this proof-of-concept system had a key limitation: the peroxidase was not robustly and specifically targeted to the external side of the plasma membrane. This is an essential requirement because living cells are not permeable to most precursor materials, and localizing reactions to the extracellular space would limit adverse effects on native intracellular chemistry[17–19]. To address this challenge, we introduced a second-generation GTCA technique that allows for precise polymer assembly by incorporating horseradish peroxidase (HRP) in a highly localized manner on the extracellular face of the plasma membrane of primary neurons while minimizing retention in the intracellular space[16] (Fig. 1a). Upon addition of polymer precursors and H_2O_2, membrane-displayed HRP facilitates oxidative polymerization on targeted neurons. The synthesized polymers form dense clusters on the surface membranes of targeted living neurons, which remain viable after polymerization[16], providing a foundation for the different applications discussed in this Perspective.

Diversifying the GTCA chemistry toolbox

To expand the capabilities of GTCA, a larger chemistry toolbox is in development involving different polymers and nanomaterials. This section outlines new approaches designed for compatibility with modern genetic engineering technologies, suitability for wild-type (non-transgenic) animals and tolerability (minimal toxicity) for living biological systems as complex and fragile as the mammalian brain.

Expanding the HRP–H_2O_2 system

Polymer precursors (monomers or dimers) with lower oxidation potential are preferable for GTCA in enabling peroxidase-catalysed oxidative polymerization to proceed even at low concentrations of H_2O_2 (≤0.05 mM)[15,16]. Another well-studied, biocompatible conductive polymer suitable for expanding the GTCA system would be polypyrrole (PPy), which may have better biocompatibility[20] than PANI and offers numerous opportunities for fine-tuning of oxidation potential and polymerization kinetics through side-chain functionalization[21] and copolymerization with other pyrrole derivatives (Fig. 1b). Notably, in electrochemical and chemical polymerization of pyrrole, the initial oxidation of the pyrrole monomer to bipyrrole is the rate-limiting step, because the oxidation potential of pyrrole is much higher

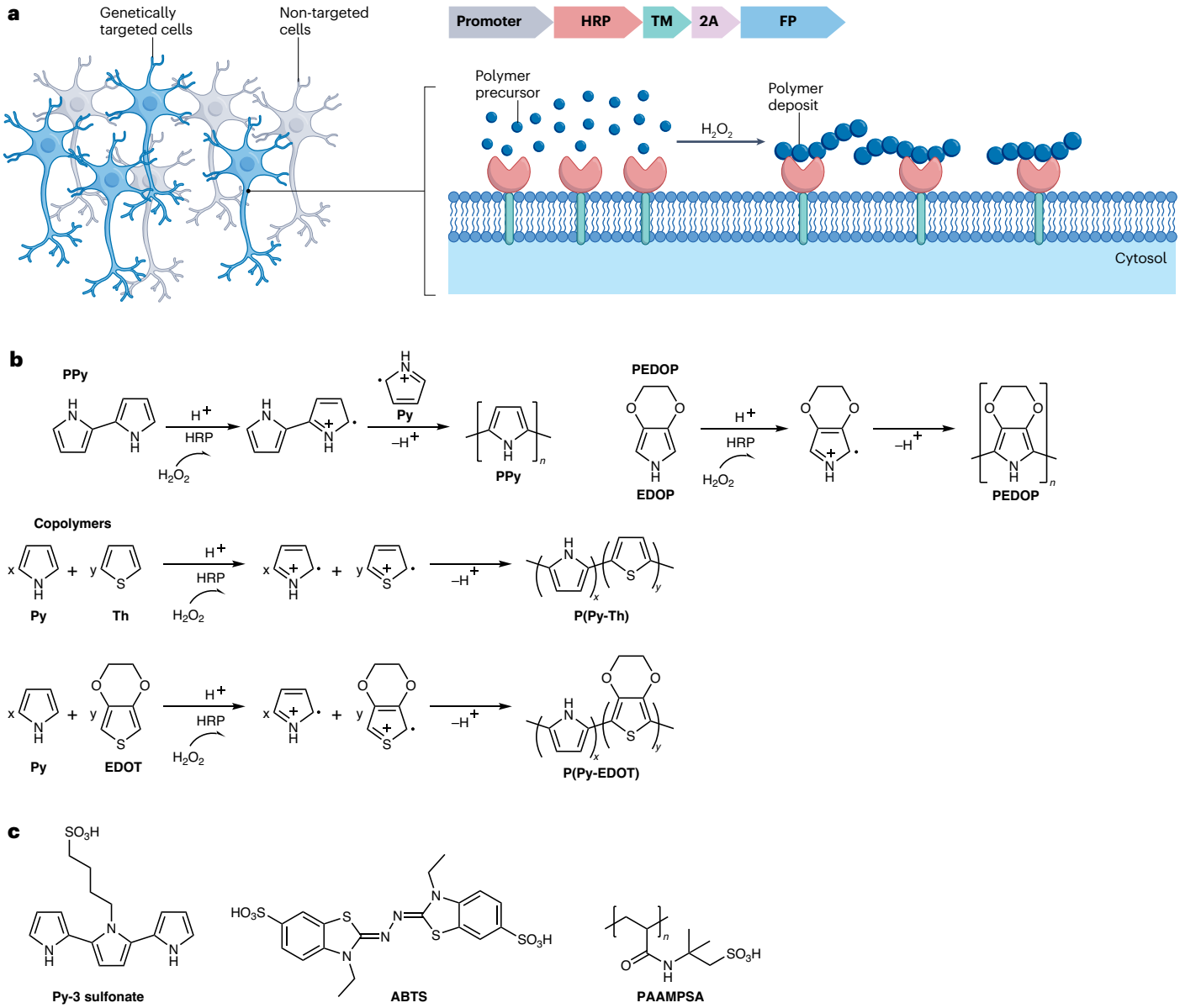


Fig. 1 | Genetically targeted chemical assembly of polymers de cellula on living cellular membranes: localization and polymerization schemata for pyrrole derivatives. a, Living systems containing genetically targeted (blue) and non-genetically targeted (grey) cells (left). DNA backbone for expressing membrane-displayed horseradish peroxidase (HRP). The construct is composed of (in one instantiation) a promoter for targeting specific cell types, followed by HRP, a transmembrane (TM) domain as the membrane-targeting anchor, 2A self-cleaving peptides and a fluorescent protein (FP). The targeted cells are expected to express membrane-displayed HRP and cytosolic FP (top right). HRP–hydrogen peroxide (H_2O_2)-catalysed polymerization is designed to occur specifically on the membrane of enzyme-targeted cells. Polymer precursors form dark-coloured aggregates deposited on the cell surface (bottom right). **b**, HRP-mediated polymerization of polypyrrole (PPy), poly(3,4-ethylenedioxypyrrole) (PEDOP) and copolymers of pyrrole-thiophene (P(Py-Th)) and pyrrole-3,4-ethylenedioxythiophene (P(Py-EDOT)). **c**, Structures of representative doping agents that may be incorporated during polymerization to increase electrical conductivity of the resulting polymers: sulfonate-containing pyrrole trimer (left); 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (middle); and poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAAMPSA) (right). EDOP, 3,4-ethylenedioxypyrrole.

than that of bipyrrole and other oligomers[22,23]. Therefore, adding bipyrrole to the polymer precursors is expected to substantially increase the reaction rate. Alternatively, another pyrrole derivative (3,4-ethylenedioxypyrrole (EDOP), where 3,4-alkylenedioxy substitution lowers the monomer oxidation potential and restricts polymerization to the 2 and 5 positions) can also be used to facilitate polymerization and reduce backbone imperfections[24,25]. Furthermore, copolymerization of PPy with other conductive polymers, such as polythiophene[26] and poly(3,4-ethylenedioxythiophene) (PEDOT)[27], may lead to new GTCA materials with tunable intermediate properties.

In another strategy to increase the conductivity of PPy synthesized under biocompatible conditions, doping agents can be added to the precursor solution and incorporated into the polymer structures (Fig. 1c). For example, self-doping of PPy can be achieved by adding alkyl sulfonate side chains on pyrrole precursors[28]. As the chain length of the conductive polymer increases, solubility in water decreases considerably, which limits the conjugation length and leads to low polymer conductivity. Using sulfonate groups not only increases the doping level but also improves polymer solubility, which further increases conductivity.

In the enzymatic synthesis of conductive polymers, another commonly used redox mediator and doping agent is 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)[29,30], an effective peroxidase substrate. ABTS can be oxidized to generate a radical cation that, in turn, chemically oxidizes pyrrole, and its sulfonate groups can be electrostatically incorporated into the PPy backbone. Another approach is to use a sulfonate acid polymer, such as poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAAMPSA)[31,32] or sodium polystyrene sulfonate (PSS)[27], as both dopant and template for PPy synthesis, which can yield water-dispersible polymers with higher conjugation length and conductivity.

Notably, incorporating a small percentage of polydopamine (PDA) into PPy can also increase polymer conductivity. Dopamine (already present in some neuron types and further promoted by the provision of precursors such as L-DOPA and enzymes such as tyrosine hydroxylase) is negatively charged and can function as a dopant; π–π stacking between PDA and PPy stabilizes charge carriers and PDA may lead to better adhesion between PPy and tissue surfaces[33–35]. Together, this diverse set of PPy precursors and doping agents may provide new functionality by enabling fine-tuning of reaction rate and polymer conductivity, thereby enabling adjustable modulation of cellular membrane properties (for example, in neurons, modulation of membrane capacitance and action potential firing).

Oxidases that do not require external H_2O_2

In the HRP–H_2O_2 GTCA system, reactions are carried out in biocompatible aqueous solutions with a low concentration of H_2O_2 (≤0.05 mM) to trigger a one-time oxidative polymerization reaction[16]. However, GTCA requires synthesized polymers to remain within the intact living system to achieve chronic modulation, and some applications may even benefit from ongoing polymer synthesis. Therefore, the potential oxidative toxicity of H_2O_2 in the long term motivates the exploration for other enzymes that can catalyse the oxidative polymerization without the need for delivery of external H_2O_2.

Within the oxidative enzyme family, besides the peroxidases, oxidases are another major class that can catalyse redox reactions by converting molecular oxygen (O_2) from air into reactive oxygen species (ROS). The first candidate is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), the only mammalian enzyme dedicated to ROS generation[36]. The NOX enzymes are transmembrane (TM) proteins that transport an electron from cytosolic NADPH to O_2 on the extracellular side of the membrane to produce a superoxide anion[36], where the unpaired electron imparts high reactivity. Certain isoforms of NOX, including NOX1, NOX2 and NOX4, can be upregulated in neurons[37]; NOX requires continuous metabolism of glucose to supply its NADPH substrate[38], and therefore, in principle, polymerization reactions can be controlled by the glucose level, although the presence of baseline NOX and glucose in unmodified cells may pose challenges for ensuring specificity of genetic targeting. In this regard, two other oxidase candidates (glucose oxidase[39] and laccase[40]) have been identified that are broadly distributed in fungi and plants but not mammals. Both oxidases can catalyse oxidative polymerization of PANI[41,42] and PPy[43,44], although their expression and function in mammalian systems need to be tested and optimized.

Conjugation of pre-synthesized materials

The first example of GTCA focused on assembly of functional materials with construction starting from the cell membrane. Although many reactions could potentially fall within the scope of such a from-the-cell or 'de cellula' regime, it is important to acknowledge that only a subset of reactions can be practically performed in this way owing to inherent limitations imposed by the underlying cellular chemistry and biology. First, all of the reactants and conditions must be biocompatible (with maintained robustness of cell structure, function and overall health in tissue after the reaction). Second, the reaction type must be based on existing genetically encodable enzyme capability, and only six groups of reactions can occur under enzymatic catalysis: redox reactions by oxidoreductases; transfer of functional groups (such as methyl or phosphate groups) by transferases; hydrolysis by hydrolases; bond cleavages by lyases; isomerization by isomerases; and covalent linkages by ligases[45]. Because of these categorical and biocompatibility limitations, many reactions cannot occur under suitable conditions in the physiological environment. Here, we summarize two alternative approaches that explore a distinct towards-the-cell or 'ad cellula' regime, which works around these limitations by attaching partially pre-synthesized polymers or particles to living cells with cell type specificity.

First, the bioorthogonal chemistry toolbox[46] can be used with GTCA to selectively introduce abiotic functional groups. Four major types of biomolecule — namely, nucleic acids, proteins, carbohydrates and lipids — have been endowed with bioorthogonal chemical moieties to be metabolized and incorporated into biological systems. Here we highlight a general enzyme-based activation strategy to unmask caged amino acids, monosaccharides and lipids to be incorporated as azido or alkyne modified metabolites (Fig. 2a). Enzyme–substrate pairs orthogonal to native biochemical reactions could be used[47,48]; for example, hydrolases such as esterase that enable the hydrolytic cleavage of ester groups to uncage acetylated azido-monosaccharides[46]. Specifically, *N*-azidoacetyl-mannosamine (ManNAz), a metabolic precursor modified with azide groups, can enter the sialic acid biosynthesis pathway and, eventually, be anchored on the cell membrane[49,50]. Once internalized, these unnatural sugars can be metabolized by native glycosyltransferases and incorporated into cell surface glycans to enable azide modification of the glycocalyx layer (including glycoproteins and glycolipids) on membranes of targeted cell types.

Moreover, azido-labelled extracellular membrane proteins could also be generated through genetic code expansion with non-canonical amino acids. For example, incorporating artificial biosynthetic pathways enables genetic targeting in this process[51–54]. Pre-synthesized polymers and nanoparticles functionalized with dibenzocyclooctyne (DBCO) groups can then be selectively anchored on the membrane through the alkyne–azide cycloaddition reaction forming a stable triazole. One potential caveat about this approach is that the unmasked molecules or metabolites, if small enough, might diffuse into neighbouring cells through gap junctions[55], resulting in decreased selectivity of surface labelling.

Delivery of functional groups to the membrane for GTCA could also be achieved using modular protein–peptide interaction systems

(leveraging the molecular strategies for gene delivery used for expressing membrane-displayed HRP in primary neurons[16]). One example that could be incorporated as a component of ad cellula GTCA would be the SpyTag–SpyCatcher system[56]; the SpyTag fragment is a small peptide (13 amino acid residues) that interacts with the SpyCatcher protein to form an isopeptide bond that is highly specific, modular and stable in living cells. Through membrane expression of SpyCatcher, and linking the SpyTag peptide to pre-assembled or partially assembled materials for GTCA synthesis such as polymers and nanoparticles, selective localization of the resulting designed structure could be achieved[57] (Fig. 2b). Alternatively, the SpyTag peptide could be fused to other membrane-expressed proteins, with the SpyCatcher conjugated onto the materials[58]. Orthogonal systems such as SnoopTag–SnoopCatcher[59] can be combined with SpyTag–SpyCatcher to enable simultaneous targeting of two different cell types and materials. We have recently demonstrated ad cellula GTCA with the streptavidin–biotin system, wherein streptavidin is expressed on the membrane to bind biotin-conjugated gold nanoparticles[16].

Genetically targeted reaction conditions

In the original demonstration of GTCA, the delivery of peroxidase-encoding vectors and monomer solutions relied on injection and subsequent diffusion, resulting in limited spatial resolution. Moreover, the HRP–H_2O_2 system is best suited for inducing a single reaction, rather than extended patterning over time. To better match the complexity and plasticity of biological assembly, here we consider other reaction conditions, with a focus on light-based approaches.

Light-driven technologies have broadly influenced different fields of science and engineering, from photolithography and 3D printing to optogenetics[60–62]. We anticipate that light-based targeting could further expand the scope of GTCA in several dimensions. First, the light source can be easily focused to a diffraction-limited spot (down to the submicron scale); in conjunction with a scanning system, the light spot can be aimed at any location of interest to form patterns with high spatial precision (to the extent compatible with light scattering, a phenomenon that can be ameliorated with multiphoton methods)[60–62]. Second, the intensity and duration of light can be tuned on-demand such that reactions are controlled with high temporal resolution. Last, light can initiate a range of photochemical (radical chemistry, fluorescence) and photophysical (photovoltaic, photothermal) responses for a multitude of applications.

3D in vivo photolithography

The state-of-the-art 3D photolithography technique, two-photon polymerization, can create arbitrary 3D nano/microstructures with sub-100 nm resolution (Fig. 3a). To write a shape, a femtosecond laser beam is tightly focused onto a photoresist block composed of photoinitiators and monomers. Multiphoton absorption by photoinitiators occurs only where light intensity is the highest, which confines polymerization to the sub-100 nm focal spot; microstructures are created through laser scanning to form predetermined geometries. Here, we describe the concept of genetically targeted in vivo photolithography, to create an arbitrary 3D conductive 'neural lace' connecting brain cells and regions (Fig. 3a). In this system, genetically encoded photosensitizers[63] that produce ROS upon illumination are used as photoinitiators that function as membrane-displayed reaction centres, facilitating photopolymerization of conductive polymer precursors. ROS produced by photosensitizers are only generated locally in situ at the illuminated area within a small distance from the cell membrane and are expected to be consumed virtually instantaneously by the monomers, thus circumventing toxicity of externally delivered H_2O_2. With a digital micromirror device or spatial light modulator-based one-photon or two-photon illumination[64],

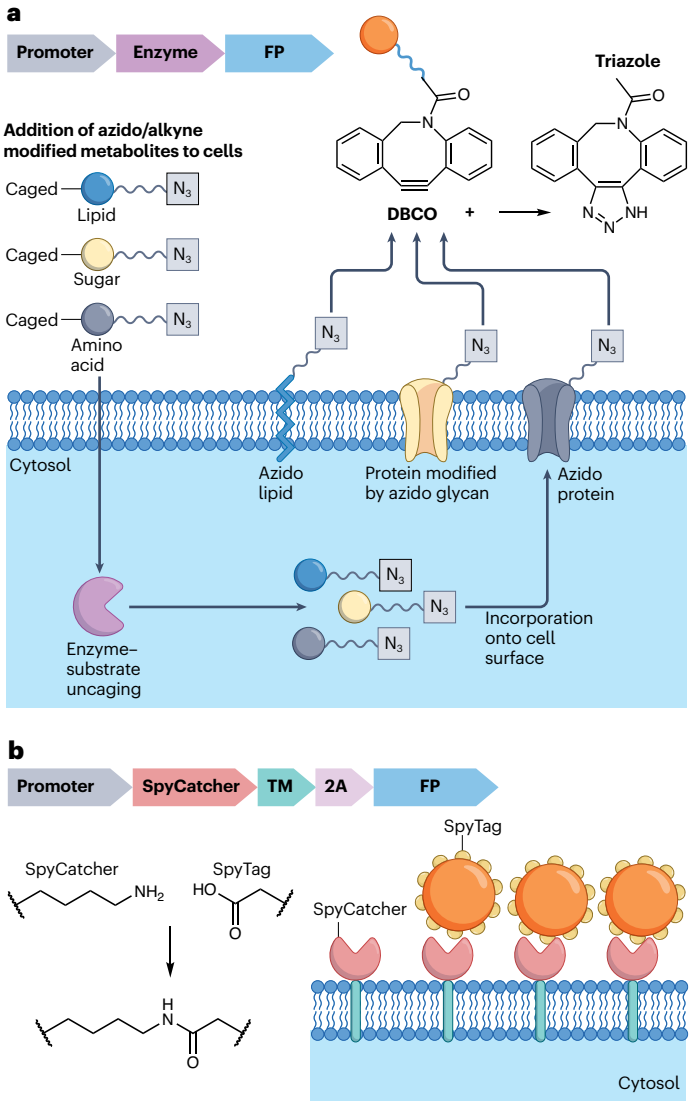
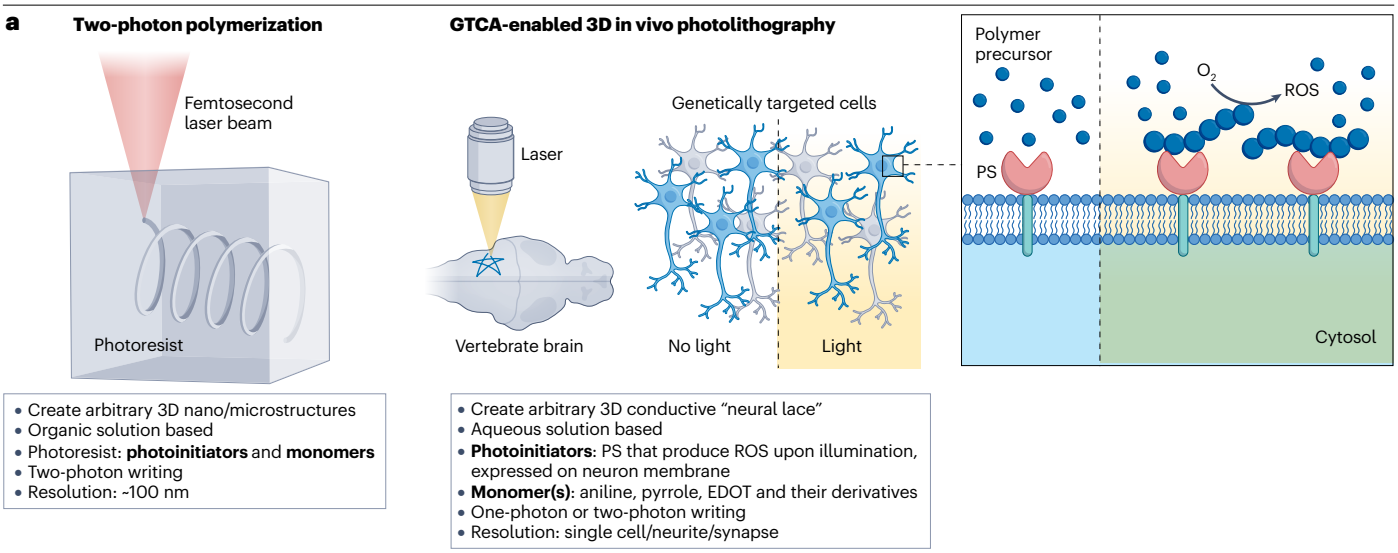
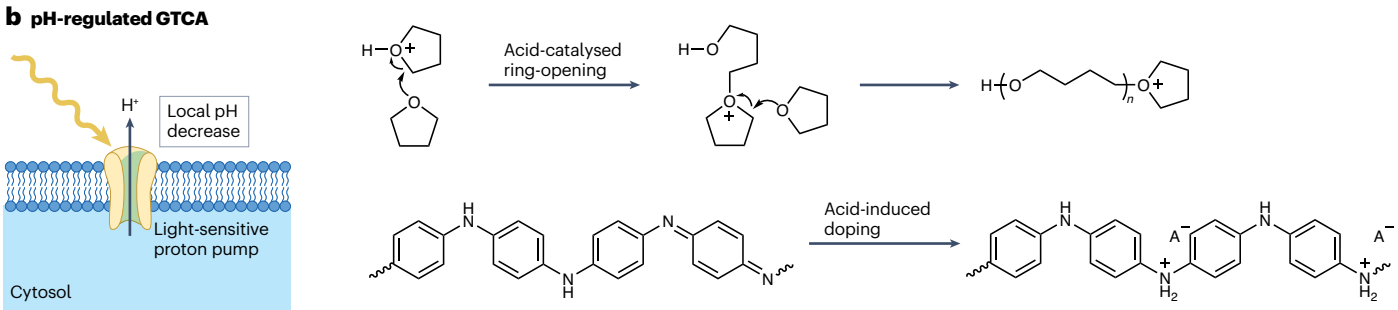
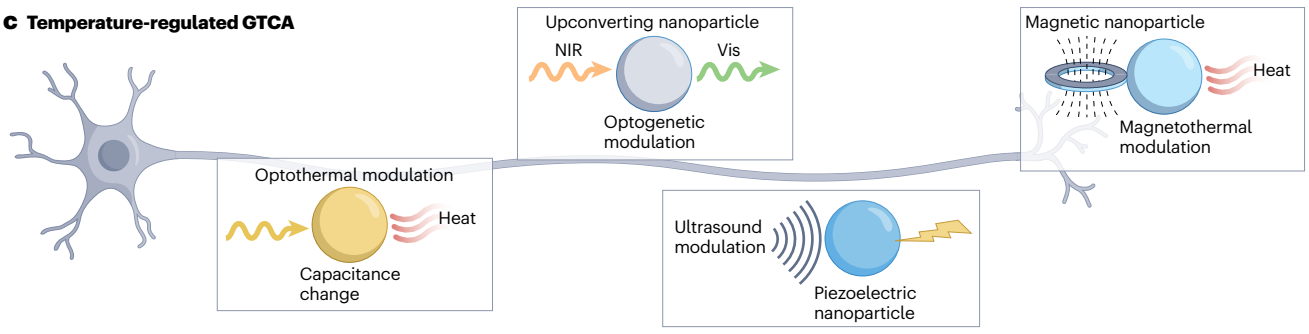


Fig. 2 | Genetically enabled ad cellula conjugation of pre-synthesized materials on living cell membranes. a, Basic DNA backbone for expressing and tracking localization of a cytosolic uncaging enzyme under control of a promoter for targeting specific cell types (top). Caged metabolites such as lipids, monosaccharides and amino acids may be internalized by cells and uncaged by specific intracellular enzymes. Unmasked metabolites may then be processed and incorporated into the lipidome, proteome and glycome. Unmasked azido sugars are enabled to modify the glycocalyx layer (including glycoproteins and glycolipids) on the cell surface with azide ($-N_3$). Materials functionalized with dibenzocyclooctyne (DBCO) groups form stable triazole with the surface azide (bottom). **b**, DNA backbone for expressing membrane-displayed SpyCatcher with a promoter for targeting specific cell types. SpyCatcher is anchored on the membrane surface with a transmembrane (TM) domain (top). SpyCatcher anchored on the cell membrane enables extracellular conjugation of SpyTag-modified materials (bottom). FP, fluorescent protein.


a
Two-photon polymerization
Femtosecond laser beam
Photoresist
• Create arbitrary 3D nano/microstructures
• Organic solution based
• Photoresist: photoinitiators and monomers
• Two-photon writing
• Resolution: ~100 nm
GTCA-enabled 3D in vivo photolithography
Laser
Vertebrate brain
Genetically targeted cells
No light
Light
Polymer precursor
PS
O_2
ROS
Cytosol
• Create arbitrary 3D conductive "neural lace"
• Aqueous solution based
• Photoinitiators: PS that produce ROS upon illumination, expressed on neuron membrane
• Monomer(s): aniline, pyrrole, EDOT and their derivatives
• One-photon or two-photon writing
• Resolution: single cell/neurite/synapse


b pH-regulated GTCA
H^+
Local pH decrease
Light-sensitive proton pump
Cytosol
Acid-catalysed ring-opening
Acid-induced doping


c Temperature-regulated GTCA
Upconverting nanoparticle
NIR
Vis
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Magnetic nanoparticle
Heat
Magnetothermal modulation
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Heat
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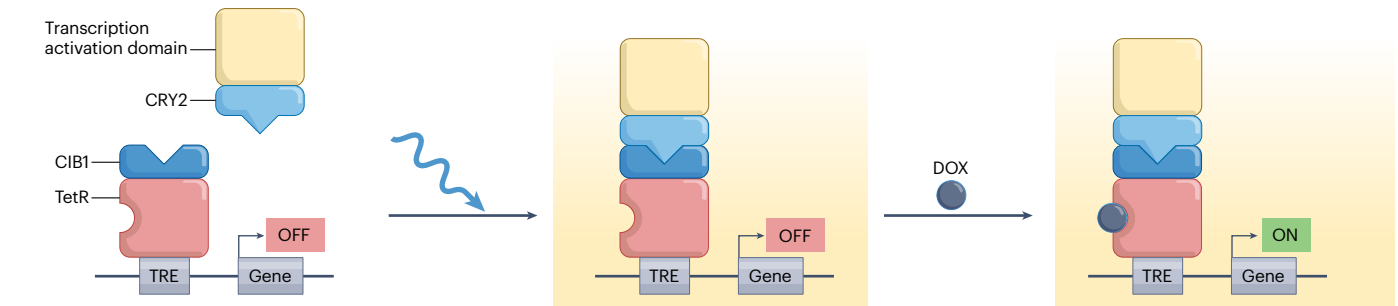
d Light-regulated gene expression for GTCA control
Transcription activation domain
CRY2
CIB1
TetR
OFF
TRE
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DOX
ON

Fig. 3 | Genetically targeted reaction conditions. a, Optogenetic genetically targeted chemical assembly (GTCA) (mode 1): genetically targeted photosensitizers (PS) and patterning of functional GTCA materials. Comparison of two-photon polymerization (left) and genetically targeted in vivo 3D photolithography (right). Bold text shows homologous components across fields. **b**, Optogenetic GTCA (mode 2): light-mediated pH change as a genetically targetable reaction condition. The working principle of microbial opsin gene-encoded light-sensitive proton pumps, such as eArch3.0 activated with 560-nm light[61,79] (left). Light-induced pH change can trigger ring-opening polymerization of tetrahydrofuran (THF) and/or doping of conductive polymers (right). **c**, Optogenetic GTCA (mode 3): light-mediated temperature change as a genetically targetable reaction condition. Shown is neuron modulation by conjugated nanotransducers capable of converting different energy modalities (including light) into temperature. The 'optothermal' mode can be complemented by magnetic and acoustic modes of temperature targeting. **d**, Optogenetic GTCA (mode 4): light-mediated expression of general reaction modulators. A light-regulated Tet-ON gene expression system. CRY2–CIB1 dimer formation between the transcription factor (TetR) and the transcription activation domain can be induced by exposure to blue light. Genetically targeted expression of any downstream gene (any reaction modulator) can then be controlled with systemic doxycycline (DOX) application. CIB1, cryptochrome-interacting basic helix–loop–helix 1; CRY2, cryptochrome 2; EDOT, 3,4-ethylenedioxythiophene; NIR, near infrared; ROS, reactive oxygen species; TRE, tetracycline-responsive promoter element; Vis, visible.

writing resolution may reach the single-cell, single-neurite or even near single-synapse level.

When initially developing this approach, we systematically analysed the genetically encoded photosensitizers that could be suitable for polymerization on living neurons, all of which are well known and have been previously validated in other settings (Table 1). The first genetically encoded ROS-generating protein reported was KillerRed[65], which produces ROS upon illumination with red light (excitation maximum of 585 nm) and has been commonly used for cell ablation[66]. However, a disadvantage of KillerRed variants is that they cannot polymerize 3,3-diaminobenzidine (DAB) for electron microscopy applications[67], suggesting lower oxidation capability than HRP. Efforts have also focused on developing genetically encoded photosensitizers based on mini singlet oxygen generator (miniSOG)[68], a fluorescent flavoprotein bound to flavin mononucleotide (FMN), which is an efficient photosensitizer. Notably, illumination of miniSOG generates sufficient singlet oxygen ($^1O_2$) to locally catalyse the polymerization of DAB for electron microscopy[66,69], and new mutants of miniSOG increase $^1O_2$ production by up to tenfold[67,70–72]. Last, a new type of genetically targeted fluorogen-activating protein (FAP), FAPdL5**, was recently developed to generate $^1O_2$ under near-infrared (NIR) illumination (669 nm)[73]. Tissue penetrability of NIR lasers opens up deep tissue applications, but whether the $^1O_2$ generated by FAPdL5** would be sufficient to trigger in situ polymerization remains to be tested. Importantly, FAPdL5** also requires incubation in the externally delivered cofactor iodine-substituted dye.

As all three photosensitizers have been expressed on cell membranes, we were able to evaluate their efficiency by comparing light doses required for cell ablation (Table 1). Of note, in all reports on membrane-targeting photosensitizers, the photosensitizers were expressed on the inner leaflet of the membrane. In contrast, in the GTCA design[74], the photosensitizers are expressed on the extracellular side; therefore, the cell ablation dose should be substantially higher and polymerization on the cell surface will be easier because ROS do not need to cross the membrane. Moreover, FAPdL5** requires much lower light intensity than KillerRed or miniSOG, indicating that FAPdL5** might be a stronger oxidizing agent for faster polymerization (Table 1). Although both miniSOG and FAPdL5** are robust choices for photopolymerization, we first used miniSOG given its track record in oxidative polymerization, which was recently successfully tested for GTCA applications by our group and others[74,75].

Going forward, to explore light-based patterning, 2D cell cultures may be used for optimizing writing/polymerization speed by tuning light intensity and composition of polymer precursor mixtures (for example, aniline and pyrrole derivatives) exhibiting different oxidation potentials. Laser scanning along lines or other defined 3D trajectories in brain tissue may be used to generate long-range conductive pathways, either to modulate intrinsic connectivity or for connection to implantable or surface electrodes for neural recording and modulation. To prevent crosstalk and current leakage from assembled conductors, conductive polymer wires may be selectively assembled with insulated coating of non-conductive polymers, wherein both polymers can be created by light-mediated GTCA. Such an approach to regulate cellular activity may be considered a mode of optogenetic GTCA (Fig. 3a), because light sensitivity is conferred in a genetically targeted way with the intent to control activity of specific cells — just as conventional optogenetics achieves with microbial opsins[61].

pH-regulated GTCA

In photolithography, distinct from photo-initiated radical polymerizations, a common photoresist chemistry of SU-8 employs a light-induced acid generator to catalyse ring-opening reactions[76]. This important concept may be translated to GTCA, where the targeted acid generator could be a genetically encoded protein that modulates juxtamembranous pH upon light delivery. Excellent candidates to assume this role would include members of the microbial rhodopsin family of proteins — specifically, the subfamily of all-in-one, single gene-encoded, light-driven proton pumps[77]. Indeed, optogenetic activation of proton-pumping rhodopsins sufficiently reduces the extracellular juxtamembranous pH to activate pH-dependent ion channels[78,79].

To leverage this capability for genetically targeted modulation of a reaction condition (here, pH) we have identified ring-opening reactions, such as tetrahydrofuran (THF) polymerization, that can be robustly catalysed by acids[80] (Fig. 3b). Microbial rhodopsins themselves can be further optimized in many ways, including tuning of light sensitivity, photocurrent magnitude and kinetics, for specific GTCA applications including THF polymerization[81], capitalizing on the detailed structural and mechanistic information that has been assembled in recent years[77]. Of note, aside from triggering acid-catalysed ring-opening polymerization before optimization, this approach could also be used for light-modulated pH changes to dope conductive polymers (Fig. 3b), a well-known method for increasing conductivity of conductive polymers by several orders of magnitude[82].

Temperature-regulated GTCA

Since the early 2010s, nanoparticle transducers have been used to convert external fields into different forms of energy (light, heat, electrical, mechanical) that can modulate neural activity[83,84]. For example, upconverting nanoparticles can convert external infrared or NIR wavelengths into local emission of visible light; NIR light allows deeper

Table 1 | Genetically encoded photosensitizers for photopolymerization

Protein	MW (kDa)	Excitation/ emission (nm)	Demonstrated applications	Cell ablation dose	Advantages	Disadvantages
KillerRed	~27×2 Dimer[65]	585/610 (ref. 65)	Cell ablation[66,69]	153 J cm^{-2} in *Caenorhabditis elegans*[128]	Expression tested in transgenic *C. elegans*, *Drosophila* and zebrafish, and mouse retina with AAV[66,69]	Cannot polymerize DAB[67]
miniSOG	~14 Monomer[68]	448/528 (ref. 68)	Cell ablation; polymerization of DAB[66,69]	Cell ablation: 280 J cm^{-2} (ref. 129) DAB polymerization: 9.72 J cm^{-2} in solution[67], 120 J cm^{-2} in *Drosophila*[130]	Can polymerize DAB, tested in multiple reports[66,69] Expression tested in transgenic *C. elegans*, *Drosophila* and zebrafish, and mouse brain[66,69] Small size[68] Mutants of miniSOG are reported to increase $^1O_2$ production by ~tenfold[67,70–72]	Short excitation wavelength may cause phototoxicity
FAPdL5**	~25 Monomer[73]	669/705	Cell ablation	~7 J cm^{-2} (ref. 131)	Deeper tissue penetration Expression tested in transgenic zebrafish[132] Efficient energy conversion	Requires 30 min–3 h incubation in iodine-substituted dye before adding monomer

AAV, adeno-associated virus; DAB, 3,3-diaminobenzidine; FAP, fluorogen-activating protein; miniSOG, mini singlet oxygen generator; MW, molecular weight; $^1O_2$, singlet oxygen.

tissue penetration and the converted visible light can optogenetically activate neurons in deep brain regions without insertion of an optical fibre[85]. However, for direct neural control, upconversion may not be the most efficient method; modern ultrasensitive microbial rhodopsins (especially the fast channelrhodopsin ChRmine) allow fast and deep optogenetic control even without upconversion[86].

Optothermal transducers, such as gold nanoparticles[87], fuzzy graphene[88] and silicon structures[89,90], can convert light into local heat, increasing the temperature by up to 10 °C, which is sufficient to directly modulate membrane excitability, leading to depolarization and activation of neurons. In addition to using light as the energy input to generate heat, other relevant signals such as magnetic fields and ultrasound waves can be leveraged. Specifically, magnetic control of neural activity has been achieved using magnetic nanoparticle heating of temperature-sensitive ion channels[91–93]. Piezoelectric nanoparticles have also been used to directly convert ultrasound waves into electricity to modulate neural activity[94,95].

By selectively conjugating nanotransducers onto living neural membranes in the GTCA ad cellula regime, these strategies can be readily adapted for neural modulation with cell type specificity (Fig. 3c). In addition, temperature represents a crucial condition for many subsequent reactions; specifically, enzymes exhibit optimal catalytic activity within narrow temperature ranges (for example, the optimum temperature of HRP is ~35 °C)[96]. Below the optimum temperature, catalytic activity increases roughly linearly with temperature, whereas above the optimum temperature, activity decreases considerably (with only 40% activity retained at 45 °C); thus, optothermal nanotransducers and magnetic nanoparticles could be used to selectively modulate polymerization.

Optically targeted gene expression

To photopattern functional materials in a genetically targeted fashion, another strategy could be to use light-regulated transcriptional promoters or enhancers to control the transcription of downstream GTCA-relevant genes[97], such as the HRP-coding gene to modulate redox conditions. For example, a modified form of the Tet-ON system (a commonly used chemically regulated gene expression tool for mammalian cells) has been developed that rendered the system responsive to blue light[98]. The original Tet-ON consists of a modified transcription factor (TetR) fused to a transcription activation domain, which can recognize and drive gene expression from a specific DNA sequence (the tetracycline-responsive promoter element (TRE)); however, transcription occurs only in the presence of the small molecule doxycycline (DOX), which binds TetR and allows association and transcription activation at the TRE[99].

In the modified photoactivatable Tet-ON system[98], the transcription activation domain and TetR are separately fused to the cryptochrome 2 (CRY2) photoreceptor and its specific binding protein cryptochrome-interacting basic helix–loop–helix 1 (CIB1), respectively (Fig. 3d). Upon blue light exposure, TetR specifically binds to the transcription activation domain through the CRY2–CIB1 light-inducible binding switch; expression of the gene of interest can thus be tightly regulated under the control of both light and DOX. The same principle has also been used to achieve optical regulation of the Gal4–UAS gene expression system in mammalian cells[100]. Notably, many other light-inducible dimerization pairs could improve this system, such as phytochrome B (PhyB)–phytochrome-interacting factor (PIF), which exhibits substantially faster kinetics and operates at long wavelength light[101].

Using such light-controlled gene expression, an early form of photolithography with bacterial cells has been achieved by optically regulating cell adhesion to substrates[102,103]. With the wide variety of light-regulated gene expression tools now available[97], any GTCA synthetic reaction with a genetically encoded catalyst component can be optically controlled.

Applications in the nervous systems

From the beginning, the long-term vision for GTCA has been one of general applicability to any animal or biological system. The first demonstration of GTCA allowed neural modulation in wild-type/non-transgenic mammalian (rodent) brains and the freely behaving *Caenorhabditis elegans* nervous system[15]. In this section, we describe several potential research and clinical applications across non-mammalian and mammalian systems and tissues.

3D photolithography in zebrafish brains

For in vivo 3D photolithography-based exploration of neural circuits, one-photon and two-photon high-resolution imaging and stimulation approaches can be used, including MultiMAP[104,105] and MultiSLM[62]. In MultiMAP, two-photon microscopy is used to perform brain-wide activity imaging with genetically encoded fluorescent Ca^{2+} indicators in live zebrafish, followed by cellular-level registration of molecular identity in fixed brains. For fast integration of single-cell imaging and stimulation we developed a method called MultiSLM, for which a wide-field high pixel-density spatial light modulator was designed for single-cell resolution NIR hologram generation. This technique enabled kilohertz 3D read–write optogenetic access to large ensembles of single neurons ($N > 1{,}000$) over millimetre spatial scales[62].

To develop versatile light-controlled polymerization while leveraging zebrafish-relevant tools such as MultiMAP and MultiSLM, a transgenic zebrafish line encoding miniSOG on all neuron membranes could be used to optimize polymerization parameters, including monomer type/concentration, light intensity and writing speed with MultiSLM. For neural modulation, distinct fish lines restricting expression of miniSOG (for example, to serotonergic neurons of the dorsal raphe)[106] alongside a nuclear-localized Ca^{2+} activity reporter (such as a GCaMP variant) in all neurons would be of substantial interest. A photopatterning approach could be used to write a conductive neural lace connecting two or more cell populations — for example, within the raphe (short range, <25 µm) and/or a pathway connecting the raphe to another relevant brain region such as the habenula (long range, 250 µm) (Fig. 4a). At the end of the experiment, MultiMAP[104] could be used to perform Ca^{2+} imaging of spontaneous or evoked neural activity and register molecularly defined cell types with the Ca^{2+} activity response map, enabling quantification and detailed understanding of modified circuit activity properties.

After patterning and insulating a conductive neural lace between brain regions, recurrent neural network models[107,108] could be used to assess altered current flow in these arbitrarily constructed neural networks in situ. Recurrent neural network models provide an estimation of the effective strength and type (such as excitatory or inhibitory) of interactions both within and across regions from experimentally observed neural dynamics. In the constructed recurrent neural network models, current flow between the two regions can therefore be estimated before and after the patterning of the neural lace from the model's recapitulation of observed activity[107,108]. Higher currents, interaction weights or synchronized firing between regions connected with the neural lace may be quantified; such measures are important because the ability to precisely and stably strengthen or weaken specific projections across the brain is crucial for meaningful control of brain states, dynamics and behaviour[77,109,110].

To further quantify neural dynamics outcomes of GTCA-created structures at single-cell resolution, brain-wide single-cell influence mapping[111] may be applied to measure how firing of one neuron causally affects spiking in its downstream partners. A fish line expressing miniSOG, a GCaMP and a channelrhodopsin such as ChRmine[62,112] in all neurons may be used alongside MultiSLM to concurrently stimulate cells on or adjacent to the neural lace. By measuring responses across the whole brain, single-cell connectivity of targeted regions can be examined[109] and potential on-target and off-target effects of the neural lace alongside behaviour can be quantified.

Modulating the mammalian peripheral nervous system

To explore the application of GTCA in the mammalian peripheral nervous system, HRP-encoding viral vectors could be readily delivered to the sciatic nerve through intraneural injection (Fig. 4b), after which solutions containing polymer precursors may be injected to deposit conductive or insulating polymers. Before and after polymer assembly, modulation of responses to electrical stimulation (such as measurement of leg movement, force generation and muscle voltage by electromyography) can be tracked, for example, using chronically implanted stretchable polymer-based electronics[113,114] that can deliver voltage pulses for electrical stimulation and record the resulting evoked action potentials. For minimally invasive approaches, a highly sensitive channelrhodopsin such as ChRmine[62,112] can be expressed in sciatic nerves to enable remote stimulation and activation threshold testing with external light sources (Fig. 4b).

For this approach, cellular specificity is a key property and can be readily demonstrated, just as in the central nervous system. The sciatic nerve includes sensory nerve fibres that are peripheral processes of neurons in the dorsal root ganglia and motor fibres that are processes of anterior horn cells of the spinal cord. Current nerve stimulation approaches with implanted electrodes cannot readily select each of these nerve types (to say nothing of their subtypes). GTCA could begin to provide physical and functional structures (that might ultimately be connected to external electronics) by targeting specific cell types through selective expression of HRP on motor or sensory neurons in the sciatic nerve[115]. Cell type specificity can be readily assessed using functional assays; with GTCA on motor neurons, changes of stimulation threshold for muscle twitching would be expected, whereas pain threshold changes would instead be expected in sensory neurons[116].

Modulating the mammalian central nervous system

Maintaining excitation–inhibition balance is crucial for nervous system function[117,118], and altered forms of this balance (such as with increased excitatory cell activity or decreased inhibitory cell activity) has been implicated in the aetiologies of autism and schizophrenia. For example, causing acute excitation–inhibition balance changes in wild-type mice could elicit or correct deficits in social behaviour[119]. Similarly, deficits in social behaviour of a transgenic mouse line lacking *Cntnap2* that exhibits autism-like phenotypes were optogenetically rescued by a temporally precise reduction in excitation–inhibition balance in the medial prefrontal cortex (either by optogenetically increasing excitability of inhibitory parvalbumin neurons, or by decreasing excitability of excitatory pyramidal neurons)[120]. In this work, excitatory (SSFO)[117] or inhibitory (SwiChR++)[121] step-function channelrhodopsins were used for highly light-sensitive optogenetic modulation.

Notably, increasing parvalbumin neuron excitability and/or decreasing excitatory pyramidal neuron excitability in the medial prefrontal cortex of *Cntnap2*-knockout mice might also be achieved using GTCA (Fig. 4c), which is compatible with social exploration testing[119]. In the simplest form of this experiment, deposition of PDAB on parvalbumin neurons and/or PANI on pyramidal neurons could be used to probe for rescue of deficits in social interaction in autism-model *Cntnap2*-knockout mice (Fig. 4c). More interestingly, the electrical connections forming and synchronizing[122] inhibitory neuronal networks could be supplemented by GTCA targeting gap junction networks linking parvalbumin neurons (Fig. 4c); in *Cntnap2*-knockout mice where excitation–inhibition balance is too high, synchronizing firing of parvalbumin neurons could be explored to restore balance in brain state and social function.

Outlook

Initial studies have demonstrated the promise of GTCA, and further studies will explore potential toxicity and long-term biocompatibility of

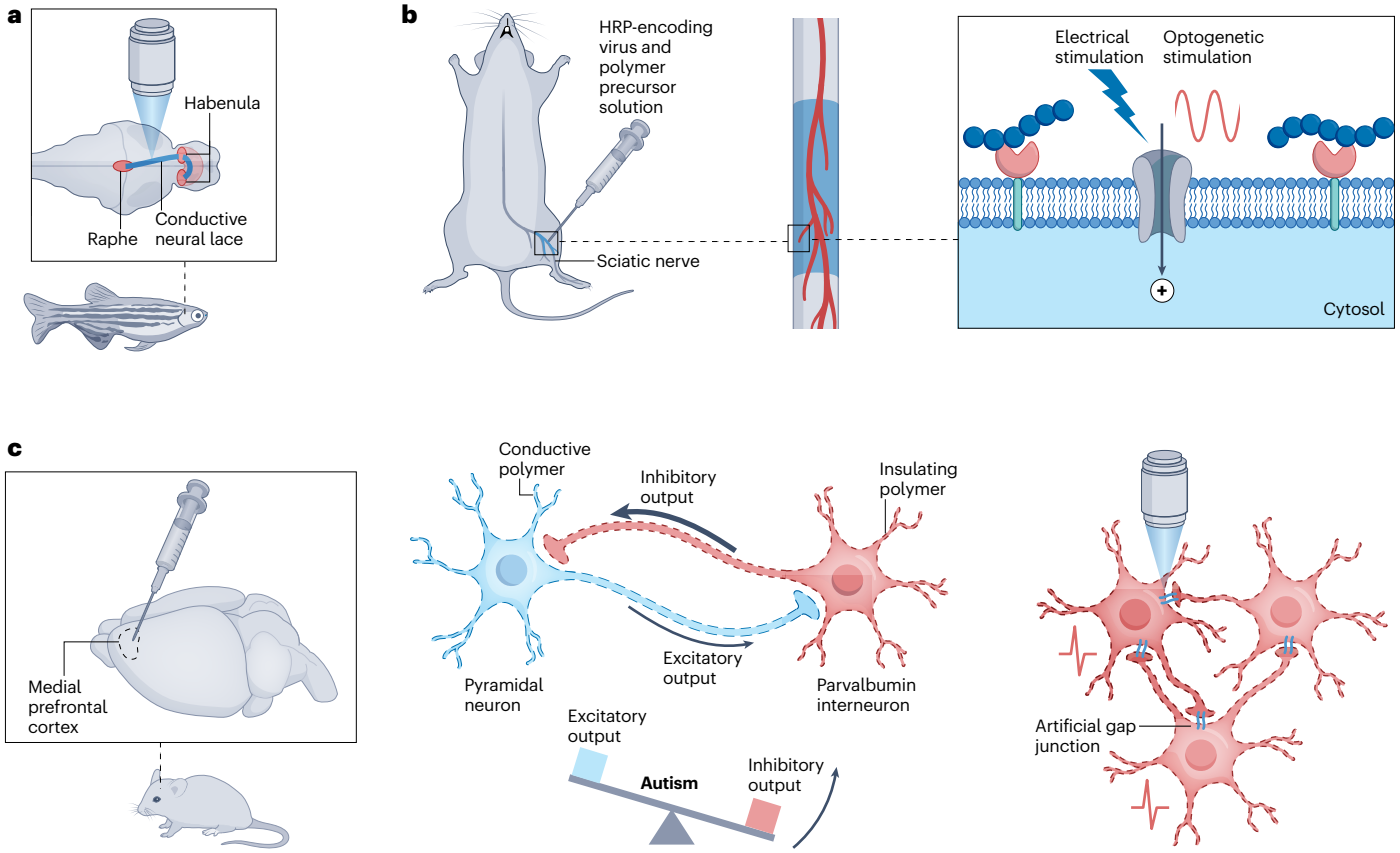

Fig. 4 | Applications from central to peripheral nervous systems. a, Genetically targeted 3D photolithography. In one configuration, a zebrafish line encodes mini singlet oxygen generator (miniSOG) on all neuronal membranes. MultiSLM (using a spatial light modulator) may be used to pattern conductive neural lace connecting structures for which net activity/current flow is thought to be behaviourally relevant, such as the left and right habenula, and/or the habenula and raphe. **b**, Peripheral nervous system modulation. Injection of horseradish peroxidase (HRP)-encoding virus and polymer precursor solutions into mouse sciatic nerves (left). Polymer deposition modulates the threshold of either electrical stimulation or optogenetic stimulation (red wave) (right). **c**, Exploring mouse models of autism. Injection of HRP-encoding virus and polymer precursor solutions into the medial prefrontal cortex in autism-model mice, such as those lacking *Cntnap2* (left). The excitation–inhibition balance governing circuit activity is mediated, in part, by excitatory pyramidal neurons and inhibitory parvalbumin interneurons, and may be modulated (or even stably corrected) by properly targeted conductive and insulating polymers (middle). Photopatterning of conductive polymers to strengthen interconnected networks such as the gap junction networks linking parvalbumin interneurons in the mammalian brain (right).

synthesized materials, as well as the physiological impact of the assembled structures on living systems. Existing strategies for increasing biocompatibility of implantable neural interfaces may be adapted to GTCA. For example, biopolymer-based coatings are known to support neuronal adhesion and reduce inflammatory response to brain implants[123], and GTCA might achieve similar effects through copolymerization with peptides, such as extracellular matrix-derived materials. Additional biodegradable polymers, including collagen, chitosan, alginate, dextran and silk, are often used as substrates for transient electronics[124,125], and could either be incorporated during in situ material assembly or provide a more biocompatible environment during reaction[126].

By integrating genetic methods with polymer chemistry and materials science, GTCA can be used to instruct specific living cells to guide assembly of functional materials. Although the structural complexity of biological systems such as the brain represents a major challenge for interface design, GTCA recruits the molecular machinery of specific cells in living organisms to construct cell-specific synthetic materials. With these and future advances, GTCA may enable construction of precisely targeted structures and interfaces within biological systems for fundamental research applications, as well as advance emerging clinical fields such as bioelectronic medicine[127] by enabling new therapeutic approaches.

Published online: 03 October 2023

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Acknowledgements

The Keck Foundation supported development of light-initiated polymerization and applications in living neural networks. The National Science Foundation Future Manufacturing Program grant (award no. 2037164) supported chemical method development for genetically targeted chemical assembly (GTCA). Both grants were awarded to K.D. and Z.B. based on the original GTCA concept and next-generation methods described previously[15]. A.Z. acknowledges support from the American Heart Association (AHA) (award no. 23POST1018301). K.Y.L. acknowledges support from the Stanford ChEM-H Chemistry/Biology Interface Predoctoral Training Program (NIH 5T32GM120007) and Bio-X Bowes Fellowship. Z.B. is a Chan Zuckerberg Biohub San Francisco investigator.

Author contributions

A.Z., Z.B. and K.D. wrote the manuscript with edits from all authors. Z.B. and K.D. supervised all aspects of the work. All authors approved the final version of the manuscript.

Competing interests

All techniques and protocols are freely available to the academic community, and the authors provide free training in genetically targeted chemical assembly (GTCA) methods at Stanford in workshops that can be accessed for registration online (https://web.stanford.edu/group/dlab/optogenetics/oil.html). Z.B. and K.D. are co-inventors of the GTCA concept used here, in intellectual property filed and owned by Stanford University.

Additional information

Peer review information *Nature Reviews Bioengineering* thanks Itaru Imayoshi, Brian Timko and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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