nature protocols

PROTOCOL

https://doi.org/10.1038/s41596-020-0367-8

Check for updates

Creating CRISPR-responsive smart materials for diagnostics and programmable cargo release

Raphael V. Gayet[1,2,3,9], Helena de Puig[2,4,9], Max A. English[1,2,9], Luis R. Soenksen[2,4,5,9], Peter Q. Nguyen[4], Angelo S. Mao[2,4], Nicolaas M. Angenent-Mari[1,2,4] and James J. Collins[1,2,4,6,7,8]✉

Materials that sense and respond to biological signals in their environment have a broad range of potential applications in drug delivery, medical devices and diagnostics. Nucleic acids are important biological cues that encode information about organismal identity and clinically relevant phenotypes such as drug resistance. We recently developed a strategy to design nucleic acid–responsive materials using the CRISPR-associated nuclease Cas12a as a user-programmable sensor and material actuator. This approach improves on the sensitivity of current DNA-responsive materials while enabling their rapid repurposing toward new sequence targets. Here, we provide a comprehensive resource for the design, synthesis and actuation of CRISPR-responsive hydrogels. First, we provide guidelines for the synthesis of Cas12a guide RNAs (gRNAs) for in vitro applications. We then outline methods for the synthesis of both polyethylene glycol-DNA (PEG-DNA) and polyacrylamide-DNA (PA-DNA) hydrogels, as well as their controlled degradation using Cas12a for the release of cargos, including small molecules, enzymes, nanoparticles and living cells within hours. Finally, we detail the design and assembly of microfluidic paper-based devices that use Cas12a-sensitive hydrogels to convert DNA inputs into a variety of visual and electronic readouts for use in diagnostics. Following the initial validation of the gRNA and Cas12a components (1 d), the synthesis and testing of either PEG-DNA or PA-DNA hydrogels require 3–4 d of laboratory time. Optional extensions, including the release of primary human cells or the design of the paper-based diagnostic, require an additional 2–3 d each.

Introduction

Smart materials that respond to biologically relevant signals play an important role in emerging biotechnologies ranging from drug delivery systems to diagnostics. These materials can provide dynamic structural supports, serve as depots for therapeutic compounds and respond autonomously to biological cues[1,2]. For example, indirect physiological signals such as pH[3] and redox states[4] have been harnessed for the targeted delivery of therapeutics to inflamed tumor microenvironments. Materials that respond to direct biological information, in the form of enzyme activity, for instance, have the potential to expand the repertoire of physiological states that can be probed and acted upon[5,6].

Nucleic acids are information-rich molecules that can be used as the input for bio-responsive materials[1]. Genomic sequences reliably identify different organisms and are routinely used to track clinically relevant markers such as antibiotic-resistance genes[7]. Similarly, knowledge of the abundances of RNA transcripts in a sample can aid clinical decision making through the prediction of treatment responsiveness[8] and microbial virulence states[9–11]. Materials that detect and respond to the presence of specific DNA or RNA sequences are therefore of particular interest in the development of next-generation diagnostics[12], as well as for applications that require the context-dependent release of therapeutics[2,13]. Incorporating DNA into materials as both a structural and information-encoding element is emerging as a promising approach to address these needs[14].

In a recent study, we demonstrated the use of programmable CRISPR-associated (Cas) nucleases as both sensors and actuators in nucleic acid–responsive hydrogels[15]. In this protocol, we provide

[1]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA. [2]Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA, USA. [3]Microbiology Graduate Program, Massachusetts Institute of Technology, Cambridge, MA, USA. [4]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA, USA. [5]Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA. [6]Synthetic Biology Center, Massachusetts Institute of Technology, Cambridge, MA, USA. [7]Infectious Disease and Microbiome Program, Broad Institute of MIT and Harvard, Cambridge, MA, USA. [8]Harvard-MIT Program in Health Sciences and Technology, Cambridge, MA, USA. [9]These authors contributed equally: Raphael V. Gayet, Helena de Puig, Max A. English, Luis R. Soenksen. ✉e-mail: jimjc@mit.edu

detailed instructions for designing and testing a representative set of CRISPR-actuated materials. We detail how this sensing platform can be used for the biomarker-dependent release of a range of cargos from hydrogel scaffolds. We also describe how CRISPR-responsive materials can be incorporated into the development of paper-based nucleic acid diagnostics with colorimetric, analog and wireless digital readouts.

Development of the protocol

Microbial CRISPR–Cas systems have been successfully adapted into powerful genetic engineering tools, owing to the facile modular programmability of their effector proteins[16]. The target sequence of a Cas nuclease is largely defined by the sequence of the gRNA to which it is complexed[17]. Beyond the canonical double-stranded DNA (dsDNA) cleavage activity characteristic of the effectors most commonly used in gene editing, the type V and VI families of Cas enzymes are able to hydrolyze single-stranded DNA (ssDNA) and RNA (ssRNA), respectively[11].

The discovery of these alternative activities has fueled the rapid development of a new class of diagnostic platforms, such as SHERLOCK[11,18–20], HOLMES[21] and DETECTR[22], that generate fluorescent or lateral flow readouts[11,22]. The last-named method uses Cas12a, a programmable nuclease guided by a single gRNA. Cas12a targeting requires both a gRNA that matches 18–24 nucleotides (nt) of the target dsDNA and a T-rich protospacer-adjacent motif (PAM) on the DNA strand opposite to the target site. Upon activation, Cas12a exhibits two distinct—but coupled—activities (Fig. 1a). When the Cas12a–gRNA complex encounters a dsDNA molecule matching the sequence of its gRNA (a 'trigger' DNA molecule), the enzyme cleaves the DNA in a sequence-specific way (termed 'targeted' or 'cis' cleavage) and remains bound to one of the resulting DNA fragments. This first cleavage event causes a protein conformational shift that enables a second, nonspecific nuclease activity (termed 'collateral' or 'trans' cleavage); the activated Cas12a–gRNA complex cleaves nearby ssDNA molecules with a very high turnover, at rates approaching the diffusion limit[22–25].

We reasoned that this programmability and intrinsic amplification mechanism would make Cas12a well suited to both the detection of biologically relevant nucleic acid cues and the subsequent large-scale actuation of materials containing structural nucleic acid elements[15]. Our approach expands the range of downstream processes that can be coupled to the original molecular recognition event from changes in physical properties of the material, such as stiffness and conductivity, to the stimulus-triggered release of bioactive cargos. To confirm the compatibility of our technology with various polymer chemistries, we tested the ability of Cas12a–gRNA to control the properties of two different materials in response to biological cues: (i) in PEG hydrogels harboring pendant groups anchored by ssDNA linkers, Cas12a–gRNA controlled the release of active cargos without altering material structure; and (ii) in polyacrylamide hydrogels crosslinked with DNA (PA-DNA), Cas12a–gRNA mediated the release of entrapped nanoparticles and cells through the complete degradation of the material (Fig. 1b).

To further expand the range of functional outputs that can be generated by a Cas12a sensor, we used the macroscale actuation of PA-DNA hydrogels to control a microfluidic paper-based analytical device (μPAD). As a demonstration of the versatility of CRISPR–hydrogel actuation, we customized the μPAD to enable the electronic detection of nucleic acids and used this feature to wirelessly transmit diagnostic data through a radiofrequency identification (RFID) tag. Cas12a–gRNA cis activity preferentially targets dsDNA substrates[22], so we incorporated reverse-transcription (RT) and isothermal amplification steps into the diagnostic workflow to enable the detection of RNA molecules. With these additional steps, we built a μPAD-based diagnostic with attomolar sensitivity for Ebola virus RNA.

Overview of the procedure

The Procedure starts with the design, synthesis and in vitro validation of Cas12a gRNAs specific to a chosen DNA target (Steps 1–18). These gRNAs form an essential part of the CRISPR sensing platform. The user then selects the architecture of the CRISPR-responsive material (e.g., PEG-DNA or PA-DNA) on the basis of the downstream application. The options for PEG-DNA gels include the release of tethered molecules such as fluorophores (Step 19A) or enzymes (Step 19B), whereas PA-DNA gels can be used to release entrapped cargos such as nanoparticles (Step 19C) or cells (Step 19D) or in μPAD diagnostics (Step 19E). Within each different option, we also provide instructions for the actuation of the synthesized CRISPR-responsive hydrogels using Cas12a–gRNA complexes and dsDNA triggers.

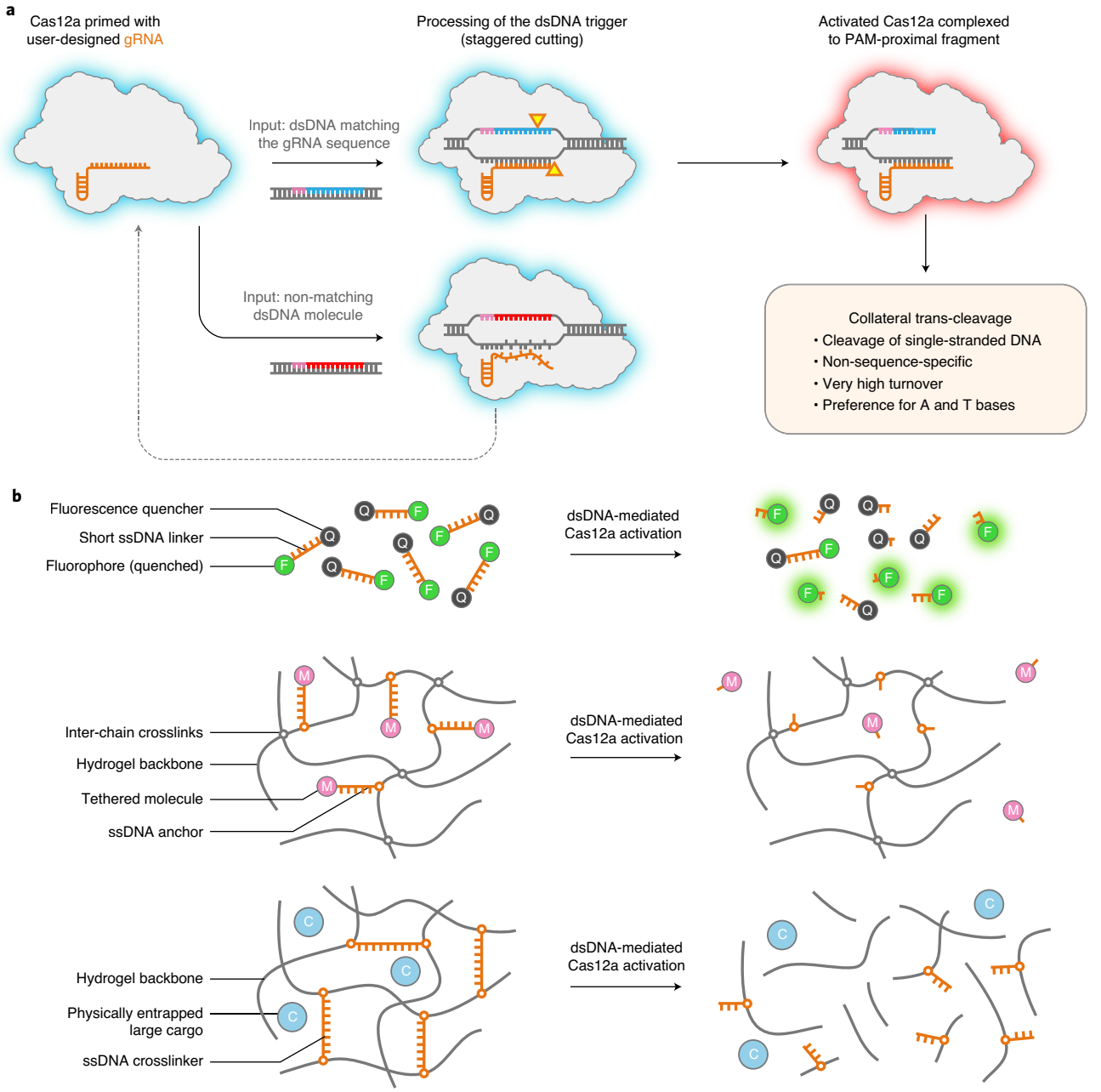


Fig. 1 | Activation of Cas12a collateral ssDNA cleavage activity by trigger dsDNA. a, Cas12a probes dsDNA molecules for complementarity based on the sequence of its gRNA (orange). Upon recognition of a matching trigger sequence (blue) flanked by a PAM (pink), the Cas12a–gRNA complex generates staggered cuts in the dsDNA molecule (yellow arrowheads) and remains bound to the fragment bearing the PAM. The resulting complex then indiscriminately cuts nearby ssDNA molecules. **b**, In this protocol, we use the trigger-induced collateral cleavage activity of Cas12a to cut ssDNA (orange) in multiple contexts: (top) to validate the activity of user-defined gRNA–dsDNA pairs, we observe the fluorescence signal generated by the dissociation of a fluorophore (F, green) from a quencher moiety (Q, black) bound by an ssDNA linker (Steps 15–18); (middle) in inert hydrogels, the cleavage of ssDNA tethers by Cas12a results in the release of molecular cargos (M, pink), such as small molecules and proteins, without altering the structure of the material (Step 19A/B); and (bottom) in materials crosslinked by ssDNA molecules, the collateral activity of Cas12a induces bulk hydrogel depolymerization and the release of enmeshed cargos (C, blue), such as cells and nanoparticles (Step 19C/D).

Comparison with other approaches

Nucleic acid–responsive materials

Typical DNA- and RNA-sensing materials rely on strand hybridization for nucleic acid detection and incorporate DNA molecules into the material itself. Interest in this approach is driven, in part, by the decreasing cost of DNA synthesis[26] and the ease with which target recognition can be predicted and programmed at the sequence level[27–29]. Methods for developing DNA-responsive materials generally

use strand displacement of crosslinking DNA elements to change the properties of the material. However, the one-to-one stoichiometry of this hybridization event can severely limit the sensitivity of these systems. As the recognition of target nucleic acids is mediated by DNA strands that also play a structural role in the material[27,29,30], decoupling the tuning of design parameters such as target sequence recognition from the gel properties can prove challenging. In our approach, the user can rapidly reprogram the specificity of Cas12a across a broad sequence space by simply altering the sequence of the gRNA, which can then be produced and validated in vitro in a few hours. This modularity greatly facilitates the process of redesigning and optimizing DNA-responsive materials to respond to different nucleic acid cues. In addition, the efficient trans cleavage activity of Cas12a–gRNA rapidly converts the detection of low concentrations of trigger DNA into macroscopic changes in hydrogel properties.

CRISPR-based diagnostics

In keeping with the previously described SHERLOCK[11,18–20], HOLMES[21] and DETECTR[22] platforms, we are able to detect both RNA and dsDNA molecules by incorporating an RT step into the workflow of a hydrogel-controlled μPAD diagnostic platform (Step 19E). The sensitivity of our assay, in the low attomolar range[15], matches that previously demonstrated for lateral flow-based SHERLOCK[11,20]. Our strategy offers the option of generating readouts that are not only informational, but also functional. The electronic modalities of our μPAD-based diagnostic turn the device into a passive component that can be easily integrated into frameworks for data processing and transmission. Although some technologies have successfully converted sequence-specific Cas binding into direct electronic signals, for example, by immobilizing the enzyme on a field-effect transistor[31], the detection of the hybridization event typically requires advanced electronic equipment that may not be widely accessible. The complexity of such a system may also increase manufacturing costs. Our CRISPR-controlled μPAD uses only low-cost materials (e.g., paper, wax), without the need for advanced equipment or specialized training, making it well suited for deployment in low-resource environments. This illustrates the ease with which this technology could be adopted and adapted by the broader scientific community.

Limitations

In our implementations of CRISPR-responsive materials, the Cas12a–gRNA complex acts as a mobile sentinel programmed to detect specific dsDNA triggers. The long-term (> 24 h) stability and activity of this complex in solution have not been investigated in detail. Applications that require sensing over longer periods are therefore outside of the scope of this work. In addition, these responsive materials are restricted to applications in which environmental conditions fall within the range tolerated by Cas12a and its gRNA; for instance, the Cas ortholog used here requires the presence of ≥5 mM Mg^{2+} cofactor in the medium and is most active at ~37 °C[15,21,22]. The stability of the DNA materials themselves is also important and can be affected by the presence of contaminating enzymes in undefined media (see 'Experimental design').

The ability to rapidly reprogram the target specificity of the system at the level of the gRNA represents a major advantage of CRISPR-responsive materials. Although this facilitates reprogramming across a broad sequence space, possible targets are limited to molecules that possess the correct PAM. The required PAM varies depending on the Cas12 ortholog[32]. The *Lachnospiraceae bacterium* Cas12a ortholog used here requires a TTTV motif (in which 'V' indicates a non-T DNA base); these may be less frequent in targets from low-GC-content genomes, for example. Efforts are ongoing to expand the target space of commonly used Cas nucleases through protein engineering[33].

Here, we present a series of demonstrations using two established material chemistries that illustrate CRISPR actuation. The literature provides many examples of how the composition, geometry and functionalization of these blank-slate materials can be modified to meet the needs of specific applications[34–37]. Although we expect our CRISPR-based actuation strategy to be generalizable across many types of hydrogels, any change to the material properties (e.g., porosity, amount of DNA linkers, geometry) can affect the response behavior[15]. For this reason, we recommend retesting the behavior of Cas12a actuation (e.g., rate of cargo release) in any new material context.

Directions for future extension of the approach

We have demonstrated the application of Cas12a to the control of material properties, but the repertoire of Cas nucleases is steadily growing. The application space of CRISPR-responsive materials

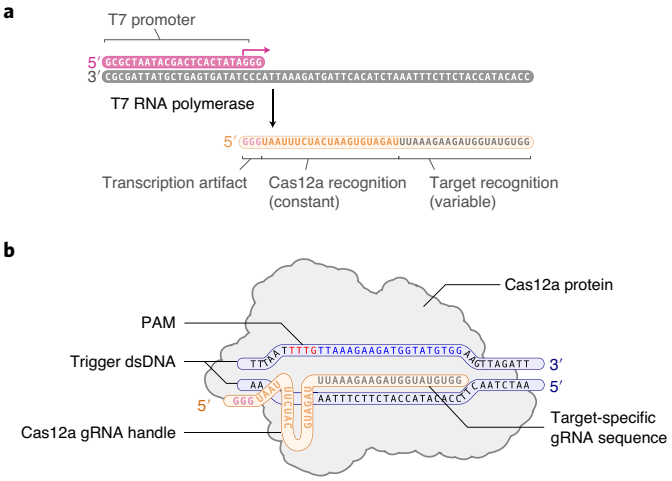


Fig. 2 | Design and synthesis of guide RNA molecules (Steps 1–14). a, The IVT of gRNAs is performed by hybridizing an adaptor (pink) with a template oligonucleotide (gray) upstream of the guide sequence. This generates a double-stranded promoter that efficiently recruits the T7 RNA polymerase. **b**, In the Cas12a ribonucleoprotein, the target-specific gRNA sequence (orange) hybridizes with the DNA target molecule. The 'TTTG' PAM (pink) is immediately 5′ of the sequence that matches the guide sequence. The sequences shown in both panels correspond to those used to activate Cas12a in response to dsDNA fragments (blue) derived from the *Staphylococcus aureus mecA* gene.

could be expanded by using the rapid, collateral degradation of ssRNA elements afforded by Cas13a[11,18,24]. Similarly, the recently described Cas14a (also known as Cas12f1) is much smaller than Cas12a but demonstrates a similar collateral cleavage of ssDNA after target recognition[38]. As the toolbox of characterized Cas enzymes grows, so does the space of potential biological inputs and material actuation strategies.

Our recent work suggests that CRISPR–Cas sensing elements can be interfaced with a range of materials. Our proof-of-concept experiments were based on a pair of well-defined synthetic scaffolds (PEG and PA) in which the DNA crosslinkers were the only functional elements. Existing examples of multi-input responsive materials suggest that CRISPR-based sensors for nucleic acid cues could be incorporated alongside other orthogonal sensing modalities within the same materials[28]. Further applications might require the use of alternative materials[39] or the functionalization of hydrogels with additional biomolecules, such as cell adhesion signals[5,35,39]. Directly tethering the functional enzyme to the material could therefore be one avenue of further investigation, as other experiments may require the Cas12a enzyme to remain co-localized with the material for prolonged periods. Despite the limited nature of the set of crosslinking chemistries that we use in this work, we expect the Cas12a-mediated actuation platform to be compatible with other similar strategies for building and functionalizing materials[34,36,37].

Experimental design

All of the experimental designs and conditions described in this article focus on a previously described set of CRISPR-responsive hydrogels (Fig. 1; ref. [15]). Whenever possible, 'Critical Step' notes have been added to specify how certain steps can be modified to adapt this protocol to other experimental situations. For users interested in investigating other material systems, the necessary controls, such as in vitro activity validations (Steps 15–18), should be performed first to verify the activity of the CRISPR reagents in anticipation of a modified experimental design.

Design and synthesis of gRNA (Steps 1–18)

One of the fundamental steps when developing CRISPR–Cas assays is the choice of a dsDNA target and the preparation of the cognate gRNA[11,18,22] (Fig. 2a). The gRNA molecule for Cas12a consists of two main parts: the constant scaffolding 'handle' region, which the Cas protein recognizes and binds to, and a user-defined spacer region that determines specificity to the target of interest (Fig. 2b). Table 2 provides examples of sequences successfully used for CRISPR-material actuation[15]. In the first part of the Procedure, we detail how to select (Steps 1–5) and synthesize (Steps 6–14) a gRNA sequence on the basis of a DNA target sequence of interest (Fig. 2a). If the target dsDNA is purchased as two strands of complementary ssDNA, we provide a brief procedure describing how to hybridize them

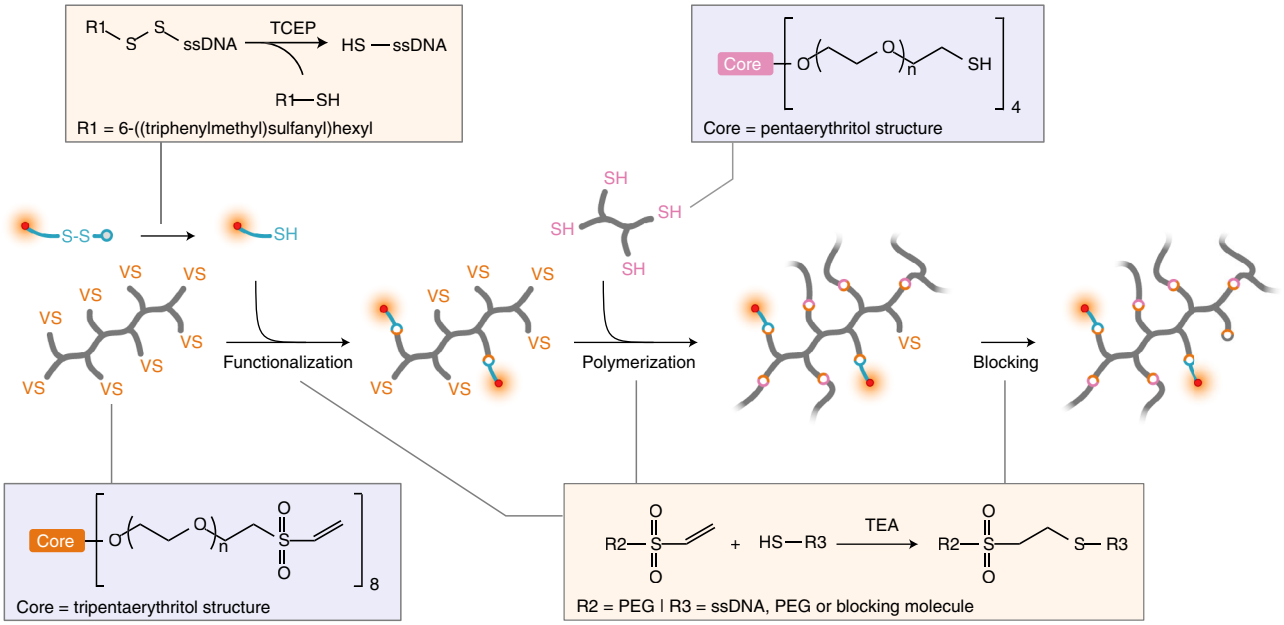


Fig. 3 | Synthesis of PEG hydrogels harboring ssDNA-tethered molecules (Step 19A). First, oligonucleotides (blue) carrying molecules of interest (red) on their 3′ termini must be treated with a non-thiol reducing agent (TCEP) to deprotect the 5′-thiol reactive groups. The 8-arm vinyl sulfone (VS)-functionalized PEG macromers (orange) undergo three successive rounds of TEA-catalyzed thiol-ene Michael additions: first with the DNA anchors to graft the cargo molecules, then with thiol (SH)-functionalized 4-arm PEG macromers (pink) to polymerize the gels and finally with a blocking thiol.

efficiently ('Reagent setup' section). Alternatively, the dsDNA can be purified from natural sources or purchased directly as dsDNA. We also detail the workflow for assessing the performance of a chosen gRNA–dsDNA pair in solution (Steps 15–18). The test is similar to the DETECTR assay described by Chen et al.[22]: Cas12a is complexed to its gRNA and subsequently mixed with the dsDNA target in the presence of a reporter probe, which consists of a fluorophore bound to a quencher via an ssDNA linker. The collateral activity of the Cas12a–gRNA complex can be observed by measuring the increase in fluorescence as the ssDNA linker is hydrolyzed (Fig. 1b).

CRISPR-responsive PEG hydrogels

In Step 19A and 19B, we detail the assembly and actuation of a class of CRISPR-responsive hydrogels for which the activation of Cas12a by a gRNA-defined dsDNA trigger results in the release of molecular cargos tethered to the hydrogel scaffold by ssDNA linkers. The structural integrity of the material itself is thus independent of the DNA oligonucleotides and is unaffected by the Cas enzyme (Fig. 1b). In the original version of this technology, we assemble PEG hydrogels decorated with labeled ssDNA anchors by first reacting sub-stoichiometric amounts of oligonucleotides onto multi-arm PEG macromers in order to functionalize the PEG backbone (Step 19A). We then use the remaining chemical handles of the PEG to polymerize the hydrogel by adding macromers with complementary chemical-end functionalization. To achieve this, we describe the use of an established Michael-type thiol–ene click chemistry (Fig. 3, Box 1), which is widely used to synthesize PEG-based biomaterials[40]. We expect this general synthesis strategy to be adaptable to other crosslinking chemistries that do not cross-react with the DNA anchors or the molecular cargos[34]. In Step 19B, we also illustrate how to graft biomolecules, such as enzymes (streptavidin–horseradish peroxidase (HRP)), onto ssDNA anchors after the polymerization of PEG hydrogels. For both options, we detail methods to track the release of cargos from the gel upon material actuation by Cas12a–gRNA in response to a defined dsDNA signal (Fig. 4).

CRISPR-responsive polyacrylamide-DNA hydrogels

In Step 19C and 19D, we outline the design and synthesis of acrylamide-DNA hydrogels for use in our Cas12a-based sensing framework by following a modified version of the protocol described by Previtera and Langrana[41]. DNA oligos functionalized with 5′-methacryl groups can be incorporated into polyacrylamide chains through a radical-catalyzed polymerization reaction (Fig. 5). Here, we

Box 1 | Example computations for PEG hydrogel synthesis

We work from a concentrated stock of each PEG macromer (4% (wt/vol)) and prepare the gels by mixing pure PEG-VS stock in TEA, diluted PEG-VS in TEA and a solution of PEG-SH in water, with the remaining volume used for other components (e.g., DNA, TCEP, water). The recipe below details how to prepare gels with PEG concentrations ranging from 1 to 2% (wt/vol); in all formulations, the molar ratio of the two types of macromers is identical (by number of cores). We alter the concentration of the diluted PEG-VS while always using the same concentration of undiluted PEG-VS stock during DNA grafting, in order to modulate the final gel concentration without affecting the functionalization.

Example calculation

Final precursor volume: V_f = (enough for a 96-well plate)
Desired PEG concentration: C_f = 1.5% (wt/vol)
For both PEG-VS and PEG-SH, make a fresh stock at a concentration of 4% (wt/vol) in TEA and water, respectively:
Chosen stock concentration C_{stock} = 4% (wt/vol)
PEG density: d = 1.1 g/cm^3
PEG aliquot mass: m = 50 mg
Volume for a 4% stock: $V = 100 \times (m/C_{stock}) - (m/d)$ = 1,204.5 µL
To make a volume V_f of gel with a concentration C_f, we use a total of three PEG solutions: one unit volume ($V_{VS1} = V_f/8$ = 62.5 µL) of PEG-VS stock that we functionalize with ssDNA, one unit volume ($V_{VS2} = V_{VS1} = V_f/8$ = 62.5 µL) of PEG-VS diluted as necessary from the stock, and two unit volumes ($V_{SH} = V_f/4$ = 125 µL) of PEG-SH diluted from a stock. Concentrations are computed as follows:
PEG-VS undiluted, for DNA grafting: $C_{VS1} = C_{stock}$ = 4% (wt/vol) in TEA
PEG-VS to adjust gel concentration: $C_{VS2} = 4 \times C_f - C_{stock}$ = 2% (wt/vol) in TEA
PEG-SH, to crosslink the gel: $C_{SH1} = 2 \times C_f$ = 3% (wt/vol) in water
This leaves four volumes ($V_f/2$ = 250 µL) for DNA, water and other aqueous components.
From these solutions, we can compute the overall molarity of crosslinks in the final hydrogels, based on the number of arms of the macromers and their molecular weight:
Number of arms on PEG-VS: K_{VS} = 8
Number of arms on PEG-SH: K_{SH} = 4
Molecular weight of PEG-VS: M_{VS} = 10,000 g/mol
Molecular weight of PEG-SH: M_{SH} = 10,000 g/mol
Molarity of vinyl sulfones in gels: $X_{VS} = 10 \times K_{VS} \times 1{,}000 \times (C_{VS1} \times V_{VS1} + C_{VS2} \times V_{VS2})/(V_f \times M_{VS})$ = 6 mM
Molarity of thiols in gels: $X_{SH} = 10 \times K_{SH} \times 1{,}000 \times (C_{SH1} \times V_{SH}/V_f)$ = 3 mM
The 3-mM excess of vinyl sulfones ensures that the DNA functionalization reaction does not compromise the subsequent reaction of the macromers with the PEG-SH by competing for functional groups. It also explains the need to block the gels with free thiols after polymerization to prevent cross-reactions with amines in downstream experiments.

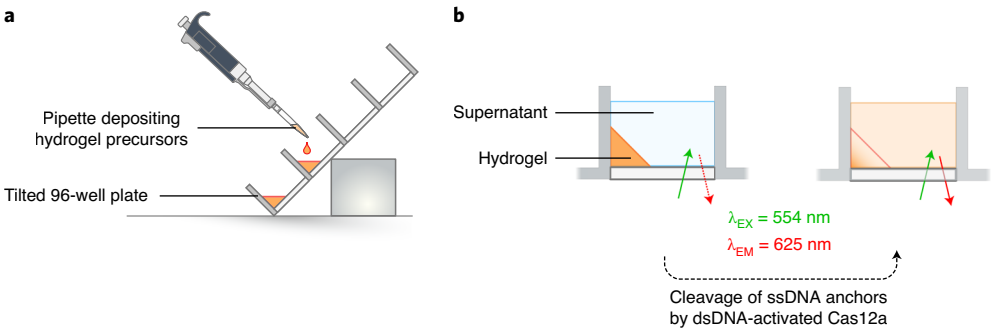


Fig. 4 | Monitoring the Cas12a-mediated release of fluorophores from PEG hydrogels (Step 19A). a, In our setup, the measurement plate is tilted on a support to ensure consistency when depositing precursors on the sides of the wells. **b**, The release of cargo can be observed in real time by measuring the fluorescence increase in the hydrogel supernatant. The fluorometer is calibrated to measure signals on the side of the well opposite from the fluorophore-loaded material, to ensure that the signal recorded is generated only by molecules released into solution.

outline the production of gel precursors with either 4% or 7% (wt/vol) acrylamide. PA-DNA hydrogels can be formed on a flat surface or in a tube to verify that the polymerization was successful and to assess their macroscopic properties. The general crosslinking protocol provided here can be used to incorporate various cargos by steric entrapment. As an example, we detail in Step 19C how to prepare gold nanoparticles (AuNPs) according to the method developed by Turkevich et al.[42], and we use these as molecular cargos in PA-DNA hydrogels (Fig. 6).

As a proof-of-concept demonstration for the use of CRISPR-responsive materials in tissue culture applications, we provide a method to entrap human cells in PA-DNA hydrogels and then release them on demand using Cas12a–gRNA and a dsDNA trigger molecule (Step 19D; Fig. 7). This protocol has been validated with two non-adherent human cell lines: primary peripheral blood mononuclear cells (PBMCs)[15] and the K562 leukemia cell line. As an illustration, we outline a

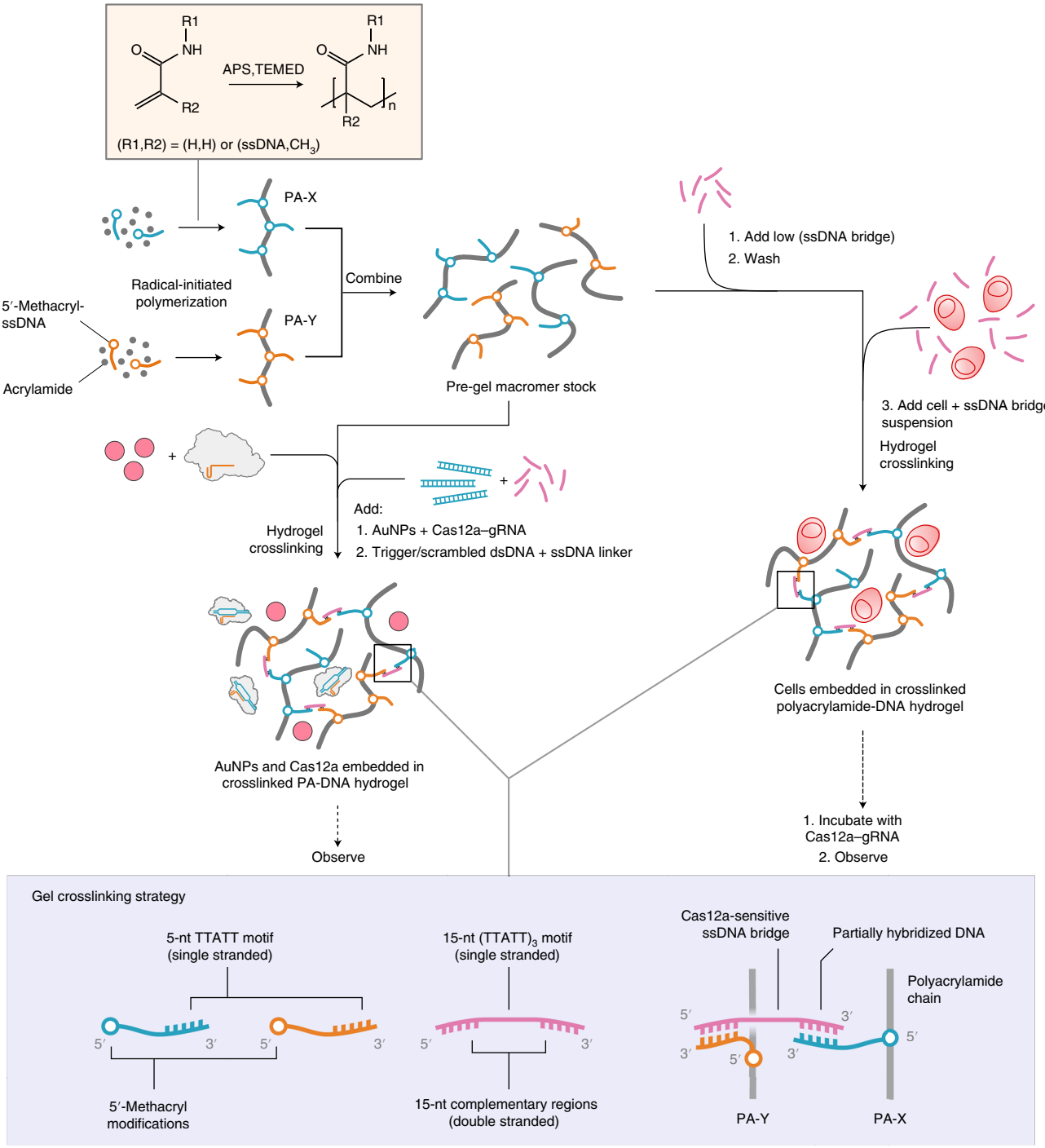


Fig. 5 | Synthesis of polyacrylamide hydrogels crosslinked by DNA molecules (Step 19B). Methacryl-functionalized DNA is incorporated into polyacrylamide chains (PA-X, PA-Y) during macromer polymerization. In one example of gel actuation (left), the Cas12a–gRNA is added to the gel precursor with the nanoparticle cargo, before the addition of dsDNA cues and ssDNA crosslinker. In another example (right), to encapsulate cells into hydrogels, a small amount of ssDNA bridge crosslinker is added to mixed macromers to thicken the pre-gel solution and minimize losses during the washing step. More ssDNA linker is then added at the same time as the cells to fully crosslink the hydrogels. Finally, the experiment is initiated by exposing the gels to gRNA-complexed Cas12a and dsDNA. Inset, Additional details of the crosslinking strategy: the two ends of the DNA bridge hybridize with distinct ssDNA anchors incorporated into polyacrylamide macromers, while the central AT-rich portion remains single-stranded and sensitive to Cas12a collateral activity.

procedure involving PBMCs. For applications involving living cells, it is important to test the growth medium for background ssDNA-cutting activity. The choice of medium may affect the long-term stability of materials containing DNA (Fig. 8).

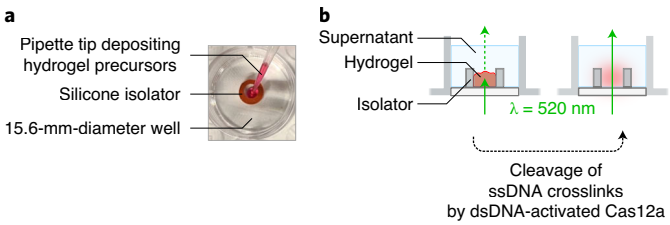


Fig. 6 | Monitoring the Cas12a-mediated release of nanoparticles through bulk PA-DNA gel degradation (Step 19C). a, The PA-DNA gel precursor is cast in a circular silicone mold placed in the center of the microplate well. **b**, Nanoparticles contained in the intact gel absorb light. Upon the detection of a trigger dsDNA by Cas12a, gel degradation causes the dispersion of the particles away from the optical path and into the surrounding solution, thereby causing a decrease in absorbance.

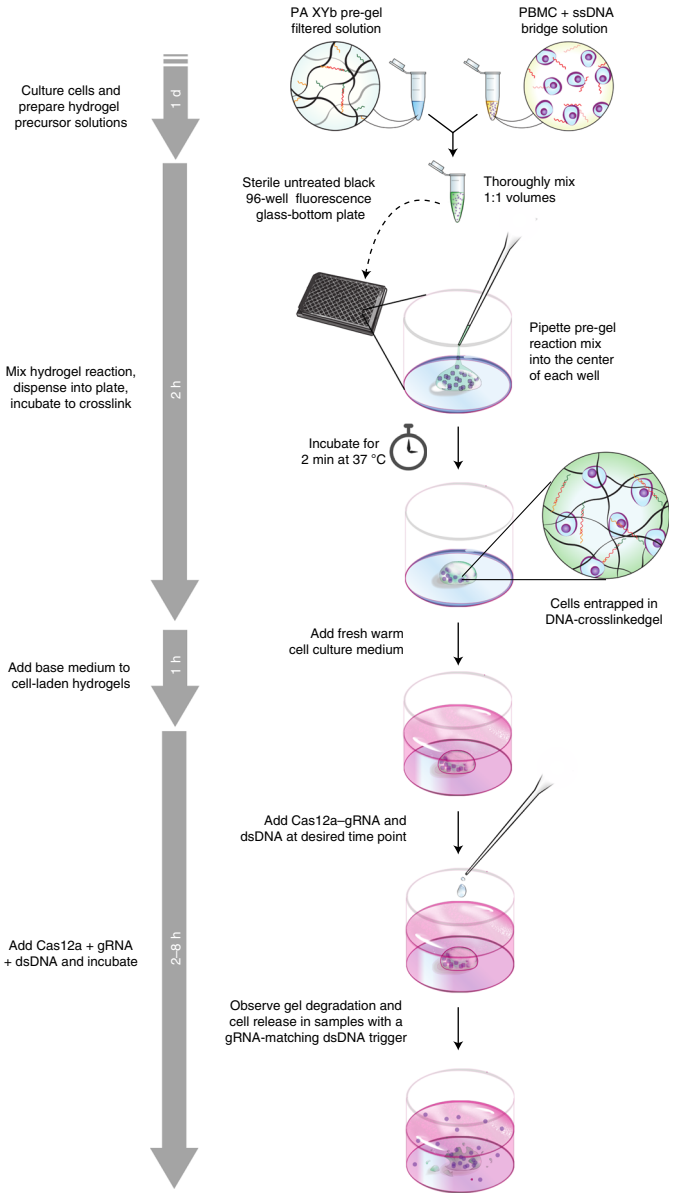


Fig. 7 | Workflow for the crosslinking and Cas12a-mediated actuation of PA-DNA hydrogels containing encapsulated cells (Step 19D). Cells are added to the PA-DNA macromers at the same time as the crosslinking ssDNA bridge, after which the solution must be dispensed quickly before the precursor thickens. Cas12a can be activated by the gRNA-defined dsDNA trigger in the cell growth medium, resulting in the degradation of the PA-DNA gel and the release of cells with minimal cytotoxicity.

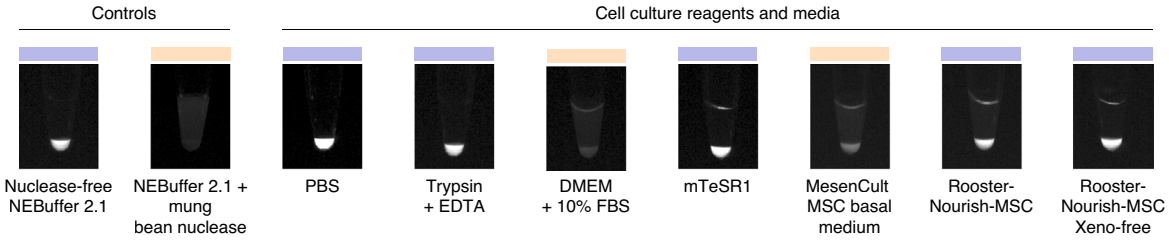


Fig. 8 | Basal ssDNA cleavage activity of cell culture media. We synthesized small PEG hydrogels harboring ssDNA-anchored cyanine dye. After washing the hydrogels, we overlaid them with various cell culture media and reagents and kept the tubes at 37 °C for 48 h before observing fluorescence. Although common cell passaging reagents (PBS, trypsin-EDTA) did not exhibit notable signs of ssDNA degradation, the performance of cell culture media was variable, which highlights the need to test one's cell culture conditions when designing CRISPR–hydrogel actuation experiments in complex media (e.g., for tissue engineering applications). Blue: no visually detectable cargo release; orange: substantial basal ssDNA cleavage activity in the absence of Cas12a nuclease. MesenCult MSC basal medium–human (https://www.stemcell.com/mesencult-msc-basal-medium-human.html); RoosterNourish-MSC (https://www.roosterbio.com/products/roosternourish-msc-kt-001/); RoosterNourish-MSC-XF (https://www.roosterbio.com/products/roosternourish-msc-xf-kt-016/).

CRISPR-mediated μPAD diagnostics

In Step 19E, we present a modified version of the protocol described by Wei et al.[43] to design μPADs for the detection of synthetic Ebola virus ssRNA (Fig. 9). We provide a protocol for the construction of the μPAD platform, which can either be used as is for visual readouts or modified further by the user (Fig. 10). To expand the capabilities of the device for ssRNA detection as well as detection of dsDNA, we detail a method to incorporate an RT–recombinase polymerase amplification (RT-RPA) step into the workflow of the CRISPR-actuated μPAD device (Fig. 9). The μPAD device used for the colorimetric detection of ssRNA and dsDNA can be modified to enable the analog electronic measurement of buffer flow through the lateral channel (Fig. 11). The basic μPAD design used for colorimetric readouts can also be modified to enable the wireless transmission of diagnostic results via an RFID tag (Fig. 12).

Materials

Biological materials

- Non-adherent cells: In the example described in this protocol (Step 19D), we use primary PBMCs purified from human samples or K562 cells (ATCC, cat. no. CCL-243, RRID: CVCL_0004), maintained in suspension culture by conventional methods as described by Masters & Stacey[44] (optional, Step 19D). Mobilized peripheral blood and leukapheresis product were anonymously collected from donors undergoing stem cell mobilization at the Massachusetts General Hospital under Institutional Review Board–approved protocol 2015P001859, with the written consent of participants. **! CAUTION** Studies involving the collection and use of human materials must be approved by the relevant regulatory board, and written informed consent of participants must be obtained. **! CAUTION** Any cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

Reagents

General reagents

- Cas12a enzyme (LbCas12a, 100 μM; New England Biolabs, cat. no. M0653T)
- Single-stranded or double-stranded DNA (ssDNA or dsDNA) containing the target sequence (Integrated DNA Technologies (IDT), custom order). If the target DNA is ordered as two single-stranded DNAs, hybridize them as described in the 'Reagent setup' section
- HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, cat. no. E2050S)
- RNA Clean & Concentrator Kit (Zymo, cat. no. R1017)
- DNAse I (RNase free; 2,000 U/mL; New England Biolabs, cat. no. M0303S)
- Mung bean nuclease (MBN; New England Biolabs, cat. no. M0250S)
- Bovine serum albumin, molecular biology grade (New England Biolabs, cat. no. B9000S)
- Custom-synthesized oligonucleotides, including primers (IDT, custom order; see Table 1)
- Ethanol (200 proof; Koptec, VWR, cat. no. 71002)
- Sodium chloride (Sigma Aldrich, cat. no. 71376)
- Tris-EDTA (TE) buffer (20×, pH 7.5; VWR, cat. no. PAA2651)
- Tris (1 M, pH 8, nuclease-free; Invitrogen, cat. no. AM9855GG)

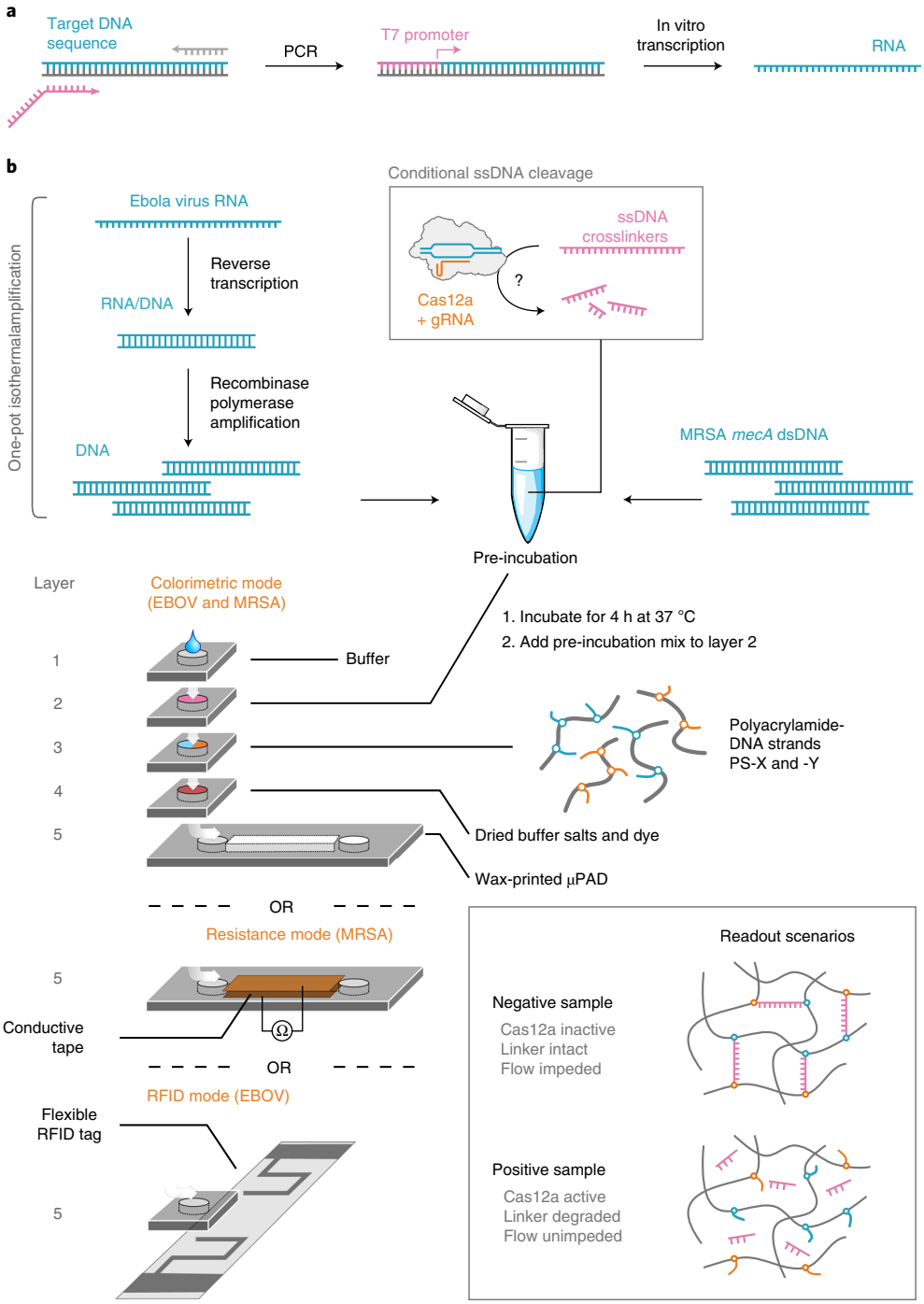


Fig. 9 | Multi-modal detection of DNA and RNA targets using CRISPR-sensitive hydrogels in paper-based fluidic devices. a, A schematic of the generation of RNA targets for the experimental validation of RNA sensors. The RNA sequence of interest (blue), ordered as a DNA element, is amplified by PCR with primers that generate a terminal T7 promoter (pink). This PCR product then serves as the template for IVT. **b**, A schematic of the workflow for the detection of DNA and RNA targets using CRISPR-responsive materials as fluidic valves in paper devices. RNA targets are first converted into a DNA signal, because Cas12a is preferentially activated by dsDNA targets. Cas12a then probes input sequences for gRNA-matching dsDNA triggers and conditionally digests the ssDNA linkers. If sufficient degradation occurs, the linkers are unable to crosslink PA-DNA hydrogels in subsequent steps. In the folded fluidic device, buffer applied to the top layer (L1) brings the product of the pre-incubation (in L2) into contact with the PA-DNA macromers (in L3), thereby polymerizing a gel within the pores of the paper, which obstructs the channel. In the case of a successful Cas12a–gRNA complex activation by dsDNA, the digestion of ssDNA bridges prevents gel formation, and buffer proceeds through L4, where it dissolves salts and dyes before reaching the lateral flow channel in L5. There, the buffer can be detected by various means: visually, or electronically, either by measuring the increase in electrical conductance through the channel (analog signal) or by recording the short-circuiting of an RFID antenna (digital signal).

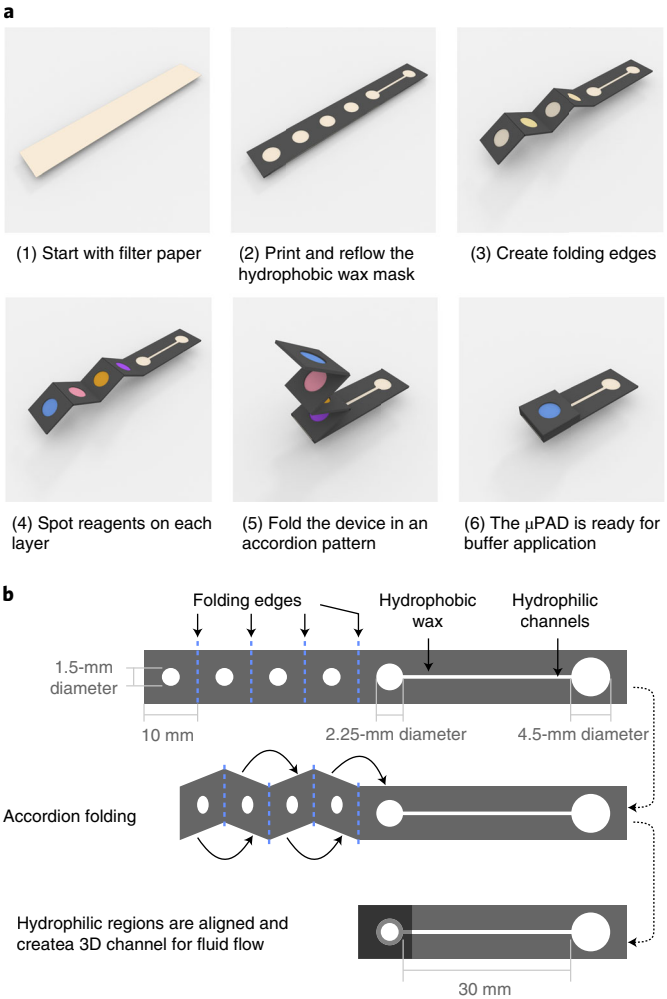


Fig. 10 | Design and assembly of the microfluidic paper-based analytical device (Step 19E(ii–xii)). a, The μPAD assembly starts with a piece of filter paper (1) to which a hydrophobic mask is applied by wax printing (2). The structure is then folded (3–5) to generate the assembled device, before buffer application (6). **b**, The stacked μPAD structure is assembled by folding paper layers on top of each other so as to align the hydrophilic regions and create a continuous channel that ends in the final lateral flow channel. **b** adapted from English, M. A. et al. Programmable CRISPR-responsive smart materials. *Science* 365, 780–785 (2019). Reprinted with permission from AAAS.

- Molecular biology-grade water (VWR, cat. no. 02-0201-1000)
- Magnesium chloride (2 M solution; Millipore Sigma, cat. no. 68475)
- Magnesium acetate tetrahydrate (Thermo Fisher Scientific, cat. no. BP215-500)
- NEBuffer 2.1 (10×; New England Biolabs, cat. no. B7202) or home-made equivalent (0.1 M Tris-HCl, 0.1 M magnesium chloride, 0.5 M NaCl, 100 μg/mL bovine serum albumin, adjusted to pH 7.9)

Optional reagents

- Triethanolamine (TEA; Sigma Aldrich, cat. no. T58300) (Step 19A/B)
- Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 0.5 M solution; Sigma Aldrich, cat. no. 646547) (Step 19A/B)
- 4-arm polyethylene glycol, pentaerythritol core, thiol-functionalized, 10 kDa (PEG-SH; JenKem Technology USA, cat. no. 4ARM-SH) (Step 19A/B)
- 8-arm polyethylene glycol, tripentaerythritol core, vinyl sulfone (VS) functionalized, 10 kDa (PEG-VS; JenKem Technology USA, cat. no. 8ARM(TP)-VS) (Step 19A/B)
- Thiol-conjugated polyethylene glycol (5 kDa, linear; Nanocs, cat. no. PG1-TH-5k) (Step 19A/B)
- Dithiothreitol (Thermo Fisher Scientific, cat. no. BP172-5) (Step 19A/B)
- Streptavidin–horseradish peroxidase (HRP) conjugate (Thermo Fisher Scientific, cat. no. 21130) (Step 19B)

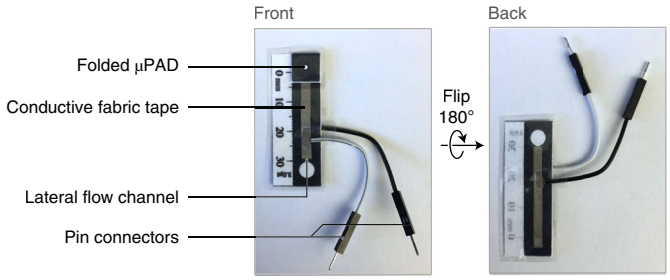


Fig. 11 | Modification of the paper-fluidic device for analog signal recording. This design is based on the μPAD used for visual readouts. Both sides of the final lateral flow channel of the folded device (layer 5 in Fig. 9) are uniformly pressed against conductive fabric tape interfaced with standard electric connectors. As a result of Cas12a-mediated valve actuation, buffer wicks through the channels and causes a measurable increase in device conductance. Adapted from English, M. A. et al. Programmable CRISPR-responsive smart materials. *Science* 365, 780–785 (2019). Reprinted with permission from AAAS.

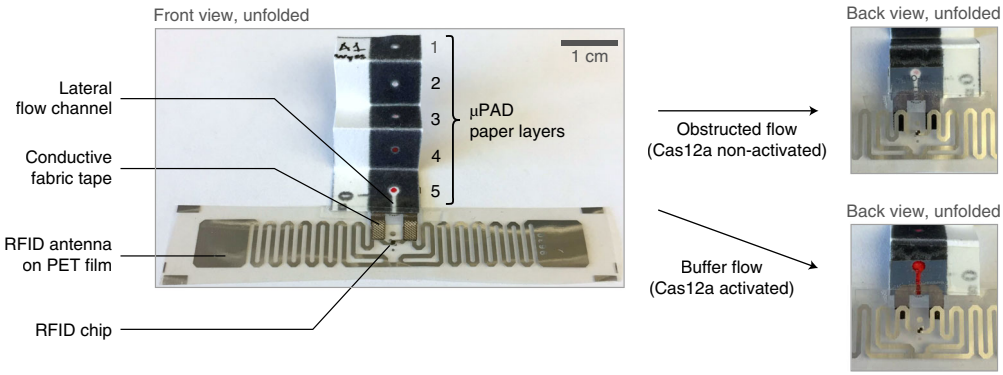


Fig. 12 | Modification of the paper-fluidic device for remote transmission of digital signals. In this design, as a result of dsDNA-dependent Cas12a–gRNA complex activation, conductive buffer reaches the final layer of the μPAD and closes a conductive loop, which short-circuits the printed RFID antenna. This results in a discrete modification of the strength of the transmitted radio frequency signal monitored at a distance by an RFID reader. Adapted from English, M. A. et al. Programmable CRISPR-responsive smart materials. *Science* 365, 780–785 (2019). Reprinted with permission from AAAS.

- Sulfuric acid (Thermo Fisher Scientific, cat. no. A300-212) (Step 19B) **! CAUTION** Concentrated sulfuric acid is corrosive and should be manipulated under a fume hood with proper protective equipment.
- Tetramethylbenzidine solution (TMB/E, ultra-sensitive, blue, HRP substrate; Millipore Sigma, cat. no. ES022-500ML) (Step 19B)
- Gold chloride trihydrate (Sigma Aldrich, cat. no. 520918) (Step 19C)
- Sodium citrate (Millipore Sigma, cat. no. W302600) (Step 19C)
- FITC-dextran (500-kDa molecular weight (MW); Millipore Sigma, cat. no. FD500S) (Step 19C) **! CAUTION** Aqua regia is highly corrosive, reacts violently with organic compounds and should be handled in a chemical hood with appropriate personal protective equipment.
- Low-molecular-weight DNA ladder (New England Biolabs, cat. no. N3233L) (Step 19C/D)
- OmniPur acrylamide (40% (wt/vol) solution; Calbiochem, cat. no. 1185-500ML) (Step 19C/D) **! CAUTION** Acrylamide is toxic. Make sure you wear proper protective equipment and process waste according to local regulations.
- Ammonium persulfate (APS; Millipore Sigma, cat. no. A3678-25G) (Step 19C/D)
- N,N,N′,N′-Tetramethylethylenediamine (TEMED; Millipore Sigma, cat. no. T9281-25ML) (Step 19C/D)
- NorthernMax-Gly Kit (Thermo Fisher Scientific, cat. no. AM1946) (Step 19C/D)
- SeaKem LE agarose (Lonza, cat. no. 50004-500G) (Step 19C/D)
- TAE buffer (50×; Bio-Rad, cat. no. 1610743) (Step 19CD)
- Heat-inactivated fetal bovine serum (FBS; Gibco, cat. no. 10082147) (Step 19D)
- Interleukin 2 (IL-2, recombinant; Gibco, cat. no. CTP0021) (Step 19D)
- Calcein Blue, AM, cell-permeant dye (Thermo Fisher Scientific, cat. no. C1429) (Step 19D)
- Ethidium homodimer-1 (EthD-1; Thermo Fisher Scientific, cat. no. E1169) (Step 19D)

Table 1 | Custom-synthesized oligonucleotides designed and used in this study

Name/description	Sequence (5′–3′)[a]	Modifications[b]
Acrydite oligo X	TTATT***CTTGTCTCCCGAGAT***	5′ Acrydite
Acrydite oligo Y	TTATT*TCACAGATGAGTATC*	5′ Acrydite
Linker-15 (i.e., L-15)	*GATACTCATCTGTGA*TTATTTTATTTTATT***ATCTCGGGAGACAAG***	NA
Linker-15-F (L-15F)	T*GATACTCATCTGTGA*TTATTTTATTTTATT***ATCTCGGGAGACAAG***	5′-6FAM
ssDNA anchor-bound Cy3 cargo for PEG gels	TTTTTATTTATCTATCTGACGA	5′-C6-thiol, 3′-Cy3
ssDNA-biotin anchor for PEG gels	TTATTATTACTATCTATTATCATTATCATT	5′-C6-thiol, 3′-biotin
mecA gRNA IVT template	**CCACATACCATCTTCTTTAA**ATCTACACTTAGTAGAAATTACC CTATAGTGAGTCGTATTAGCGC	NA
ermA gRNA IVT template	**TCCATCTCCACCATTAATAG**ATCTACACTTAGTAGAAATTACCCT ATAGTGAGTCGTATTAGCGC	NA
ermC gRNA IVT template	**ACATGCAGGAATTGACGATT**ATCTACACTTAGTAGAAATTACCC TATAGTGAGTCGTATTAGCGC	NA
spa gRNA IVT template	**ACAAAGCTCAAGCATTACCA**ATCTACACTTAGTAGAAATTACCCT ATAGTGAGTCGTATTAGCGC	NA
vanA gRNA IVT template	**CTCGACTTCCTGATGAATAC**ATCTACACTTAGTAGAAATTACCCT ATAGTGAGTCGTATTAGCGC	NA
T7 promoter sequence for hybridization	GCGCTAATACGACTCACTATAGGG	NA
Scrambled *mecA* dsDNA trigger	TAGTAGTGATTATGTTAGATAGTGAATAGGTTTAATGTAT	NA
Fluorophore-quencher reporter (FQ)	TTATT	5′-6FAM, 3′-IowaBlackFQ
mecA dsDNA trigger	TTTAATTTTGTTAAAGAAGATGGTATGTGGAAGTTAGATT	NA
ermA-dsDNA trigger	GCTTTGGGTTTACTATTAATGGTGGAGATGGATATAAAAA	NA
ermC dsDNA trigger	TAATATTGTTTAAATCGTCAATTCCTGCATGTTTTAAGGA	NA
spa dsDNA trigger	TTCACCAGTTTCTGGTAATGCTTGAGCTTTGTTAGCATCT	NA
vanA dsDNA trigger	ACGGAATCTTTCGTATTCATCAGGAAGTCGAGCCGGAAAA	NA
ZEBOV gblock (dsDNA)	GTGCGCGTTCCTACTGTATTTCATAAGAAGAGAGTTGAACC ATTAACAGTTCCTCCAGCACCTAAAGACATATGTCCGACCTTGA AAAAAGGATTTTTGTGTGACAGTAGTTTTTGCAAAAAAGA CCACCAGTTAGAAAGTTTAACTGATAGGGAATTACTCCT ACTAATCGCCCGTAAGACTTGTGGATCAGTAGAACAACAAT TAAATATAACTGCACCCAAGGACTCG	NA
ZEBOV RPA-1 (forward primer)	CTACTGTATTTCATAAG AAGAGAGTTGAACC	NA
ZEBOV RPA-2 (reverse primer)	AATTGTTGTTCTACT GATCCACAAGTCTTAC	NA
PCR primer for the ZEBOV gblock, with T7 for IVT	GCGCTAATACGACTCACTATAG GGTGCGCGTTCCTACTGTATT	NA
qPCRT-ZEBOV-R (RT reverse primer)	GAGTCCTTGGGTGCAGTTATATT	NA

All of the molecules are single-stranded unless described otherwise. NA, not applicable. [a]Bold and italic regions indicate complementarity between the Acrydite oligo X and Linker-15 or Linker-15-F of the hydrogel; italic-only regions indicate complementarity between the Acrydite oligo Y and Linker-15 or Linker-15-F of the hydrogel; bold-only regions indicate sequences in the gRNA templates that correspond to the pathogen detection targets *mecA*, *ermA*, *ermC*, *spa* and *vanA*. [b]Integrated DNA Technologies naming conventions.

- RPMI 1640 Medium, GlutaMAX supplement (Thermo Fisher Scientific, cat. no. 61870036) (Step 19D)
- Opti-MEM reduced-serum medium, GlutaMAX supplement (Thermo Fisher Scientific, cat. no. 51985091) (Step 19D)
- Dulbecco's modified PBS, without calcium or magnesium (Gibco, cat. no. A1285601) (Step 19D)
- Superscript IV Reverse Transcriptase (Invitrogen, cat. no. 18090010) (Step 19E)
- TwistAmp Basic (TwistDx, cat. no. TABAS03KIT) (Step 19E)
- Silver nanoparticle ink (Mitsubishi NanoBenefit 3G Series) (Step 19E)
- Red food dye (McCormick) (Step 19E)

Equipment

- Thermal cycler (Bio-Rad, model no. T100)
- Gel imager (Syngene USA, model no. G:Box mini 9)
- Microplate reader (BioTek, Synergy NEO HTS model or Molecular Devices, model no. SpectraMax M5)

- Freeze-dryer (SP Scientific, Freezemobile model) (optional, Step 19E)
- Inverted microscope (Thermo Scientific, model no. EVOS FL) (optional, Step 19D)
- Digital stirring hot plate (Cole Parmer, model no. IKA C-Mag HS)
- Benchtop microcentrifuge (Corning, LSE model)
- Wax (solid ink) printer (????, model no. Xerox, model no. Phaser 8560))optional, Step 19E)
- Hot press (Cricut, EasyPress model) (optional, Step 19E)
- Modified inkjet photo printer (Canon, model no. PIXMA iP7220), modified as described by Lee et al.[45], with (Canon, model no. QY6-0082) printhead and refillable cartridges (optional, Step 19E)
- Benchtop digital multimeter (Keysight, cat. no. 34411A) (optional, Step 19E)
- UHF-RFID antenna (Sparkfun, cat. no. WRL14131) (optional, Step 19E)
- Simultaneous RFID reader (SparkFun, model no. M6E Nano SEN14066) (optional, Step 19E)
- Arduino Uno Rev3 microcontroller (Arduino, cat. no. 8058333490090) (optional, Step 19E)
- Incubator (37 °C, VWR model 1545)
- Orbital rocker (Boekel, model no. 201100)
- Automated cell counter (Thermo Fisher Scientific, cat. no. A27974) (optional, Step 19D)
- Biosafety level 2 tissue culture facility with laminar flow hood and standard incubator (37 °C, 5% CO_2) (optional, Step 19D)
- Microvolume spectrophotometer (Thermo Scientific, NanoDrop model no. ND2000C)
- Rainin Pipet-Lite LTS Pipettes (L-1000XLS+, L-200XLS+, L-20XLS+, L-2XLS+; Mettler Toledo, cat. nos. 17014382, 17014391, 17014392, 17014393)
- Rainin pipette tips (LTS 1,000 µL 768/4 SS-L1000, LTS 250 µL 960/5 SS-L250 and LTS 10 µL 960/5 SS-L10; Mettler Toledo, cat. nos. 17007090, 17005875, 17005873)
- Conical centrifuge tubes (15 mL and 50 mL; Corning, cat. nos. 352097, 3520980)
- Microplates (384 wells, low volume, non-binding surface, black with clear flat bottom (Corning, cat. nos. 3544)
- Microplates (96 wells, black with clear flat bottom, non–tissue culture treated (Corning, cat. no. 3631)
  ▲ **CRITICAL** Tissue culture–treated microplates are hydrophilic and can make hydrogel casting more difficult.
- Microplate aluminum sealing tape (Corning, cat. no. 6570)
- Press-to-Seal silicone isolators (round, 2.0-mm diameter, 1.7-mm depth; Thermo Fisher Scientific, cat. no. GBL665208-25EA) (optional, Step 19C)
- Microseal 'B' Seals (Bio-Rad, cat. no. MSB1001)
- Cell culture plate (24 wells, flat bottom with lid, tissue culture treated; Corning, cat. no. 3526)
- Sterile disposable biopsy punch (5 mm, Tru-Punch; Sklar, cat. no. 96-1120) (optional, Step 19C)
- PCR tubes with flat caps (0.2 ml, Axygen; Fisher Scientific, cat. no. PCR02C)
- Microcentrifuge tubes (1.7 mL; VWR, cat. no. 87003-294)
- Ultrahigh-frequency (UHF) RFID tags (Sparkfun, cat. no. WRL-14147) (optional, Step 19E)
- Centrifugal spin filters (Amicon Ultra, 0.5 mL, 10-kDa MWCO; Millipore Sigma, cat. no. UFC501008)
- Nickel/copper conductive fabric tape (Laird Technologies, cat. no. 87580) (optional, Step 19E)
- Chromatographic filter paper (Whatman grade 1; Thermo Fisher Scientific) (optional, Step 19E)
- Vacuum filter/storage bottle system (500 mL, 0.22-µm pore size; Corning, cat. no. 431097)
- Membrane filter unit (MilliporeSigma Steriflip SCGP00525)
- Lyophilizer (Labconco, model Console) (optional, Step 19E)

Reagent setup

Hybridization of two single-stranded target DNAs into dsDNA

If the target DNA is ordered as two complementary ssDNA molecules, hybridize them as follows:

1 Dilute each of the ssDNA molecules to be hybridized (ssDNA1 and ssDNA2) to a final concentration of 100 µM in nuclease-free water.

2 Prepare the hybridization mixture by combining the reagents as follows:

Reagent	Volume (µL)
5 M NaCl	0.5
10× TE buffer	5
ssDNA 1 (100 µM)	22.5
ssDNA 2 (100 µM)	22.5
Total	50.5

3 Anneal the ssDNA target sequences by performing a 5-min denaturation and then slowly cool the reaction to 4 °C in a PCR thermal cycler. For the slow cooling of the samples, adjust the ramp rate to 0.04 °C/s. The hybridized DNA can be used immediately or divided into aliquots and stored at −20 °C for up to several months.

FITC-dextran stock solution (optional, Step 19B)
Dissolve FITC-dextran particles in nuclease-free water to a final concentration of 2.5 mg/mL. Make aliquots of this stock in microcentrifuge tubes and store them at −20 °C until needed.

Polyacrylamide hydrogel reagents (optional, Step 19C)
Dilute the 50× TAE buffer and mix it with magnesium acetate to make a 10× TAE, 125 mM magnesium acetate solution, which will be a 10× concentration of the reaction buffer. The 10× TAE buffer can be stored at 4 °C for 2 weeks. Prepare fresh 20% (vol/vol) TEMED and 2% (wt/vol) APS solutions in nuclease-free water before each polymerization reaction.

24-well plates for acrylamide gel release assays (optional, Step 19C)
Use a 5-mm-diameter tissue biopsy punch to cut Press-to-Seal silicone isolators into rings such that the wells of the isolators are at the center. Using tweezers, stick the silicone isolators into the 24-well plates so that the center of each isolator aligns with the middle of the respective well. Confirm that the isolators do not obstruct the optical path of the plate reader by measuring the absorbance of each well at an arbitrary visible spectrum wavelength; the absorbance value should be identical to that of an empty well. Do not use obstructed wells.

Cell culture media (optional, Step 19D)
Make R10 medium by combining RPMI 1640 with 1× GlutaMAX supplement, 10% (vol/vol) FBS and 2 ng/mL recombinant IL-2. Make O10 medium by mixing Opti-MEM reduced-serum medium with 10% (vol/vol) FBS. Prepare sterile working solutions of 200 mM magnesium chloride by diluting and filtering a 2 M magnesium chloride stock. Sterile media can be stored at 4 °C for up to 2 weeks. Warm all media that will be in contact with cells in a 37 °C bath before use.

Procedure

Designing, producing and testing gRNAs and dsDNA triggers ● Timing 2 d

1 *Designing the guide RNA (Steps 1–5).* Choose an 18- to 24-nt target sequence within the marker that is immediately in 3′ of a TTTV (where V indicates a non-T DNA base) PAM region that is specific for Cas12a. The target sequence should not contain additional TTTV motifs.
▲ **CRITICAL STEP** Several resources are publicly available to help in the design of efficient gRNAs: Benchling (https://benchling.com/pub/cpf1), Broad Institute GPP sgRNA Designer (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), and Zhang Lab—Guide Design Resources (https://zlab.bio/guide-design-resources).
▲ **CRITICAL STEP** dsDNA markers can be bound and detected by the Cas12a–gRNA complex with high sensitivity. Cas12a is also able to bind ssDNA in a sequence-specific manner, but the level of activation of collateral trans-cleavage is lower than with dsDNA (see Fig. 2 of ref. [22]). ssRNA markers require an RT and amplification step to convert the signal into dsDNA (see Step 19E(xix)).
▲ **CRITICAL STEP** Different Cas enzymes require distinct PAM sites. It should be noted that the gRNA design and assembly described here use a negative-sense template and a short T7 promoter adaptor oligo for the production of the gRNA through in vitro transcription (IVT, Fig. 2a). Extension of the oligo is not needed because T7 RNAP can actively transcribe dsDNA or ssDNA templates[46].
▲ **CRITICAL STEP** As a negative control in all downstream Cas12a activation experiments, we recommend using a synthetic, sequence-randomized ('scrambled') dsDNA molecule.

2 Start the gRNA design (5′ to 3′) by taking the reverse complement of a nuclease-specific gRNA direct repeat (the bold italic sequence in the gRNA in Table 2). This is the same for all the LbaCas12a gRNAs used in our experiments.
▲ **CRITICAL STEP** Different Cas enzymes may require distinct direct repeats in the gRNA.
? TROUBLESHOOTING

3 In the position immediately upstream (5′) of the sequence from Step 2, include the reverse complement of the 18- to 24-nt target-specific RNA sequence as determined in Step 1 (shown in bold in Table 2).

Table 2 | Design of gRNA molecules and their transcription templates for five different example DNA target sequences derived from staphylococcal antibiotic-resistance genes

Target name	gRNA IVT template (5′–3′)	gRNA (5′–3′)	Target (5′–3′)
mecA	**CCACATACCATCTTCTTTAA** ATCTACACTTAGTAGAAA TTACCC*CTATAGTGAGTCGTATTAGCGC*	GGG***UAAUUUCUACUAAGUGUAGAUUU AAAGAAGAUGGUAUGUGG***	TTTAATtttg**TTAAAGAAGATGGTATGTGG** AAGTTAGATT
ermA	**TCCATCTCCACCATTAATAG**ATCTACA CTTAGTAGAAATTACCC*TATAGTGA GTCGTATTAGCGC*	GGG***UAAUUUCUACUAAGUGUAGAUC UAUUAAUGGUGGAGAUGGA***	GCTTTGGGttta**CTATTAATGGTGGAGATGGA** TATAAAAA
ermC	**ACATGCAGGAATTGACGATT**ATCTACACTTA GTAGAAATTACCC*TATAGTGAGTCGTATTAGCGC*	GGG***UAAUUUCUACUAAGUGUAGAUA AUCGUCAAUUCCUGCAUGU***	TAATATTGttta**AATCGTCAATTCCTGCATGT** TTTAAGGA
spa	**ACAAAGCTCAAGCATTACCA**ATCTACACTTAGT AGAAATTACCC*TATAGTGAGTCGTATTAGCGC*	GGG***UAAUUUCUACUAAGUGUAGAUUG GUAAUGCUUGAGCUUUGU***	TTCACCAGtttc**TGGTAATGCTTGAGCTTTGT** TAGCATCT
vanA	**CTCGACTTCCTGATGAATAC**ATCTACACTTAGT AGAAATTACCC*TATAGTGAGTCGTATTAGCGC*	GGG***UAAUUUCUACUAAGUGUAGAUG UAUUCAUCAGGAAGUCGAG***	ACGGAATCtttc**GTATTCATCAGGAAGTCGAG** CCGGAAAA

Lowercase: PAM; bold: target sequence; italics: T7 promoter; bold & italic: Cas12a gRNA handle.

4 Check that the PAM (lowercase in Table 2) is immediately on the 5′ end of the target DNA sequence that matches the gRNA (Fig. 2b).

5 For the production of the gRNA in vitro using T7 transcription (Steps 6–14), add the reverse complement of the T7 promoter at the 3′ end of the designed sequence. We included the three guanines present in the T7 promoter consensus sequence (Table 2 and Fig. 2a) to increase transcription yield. Order the resulting T7 adaptor oligonucleotide and matching gRNA template oligonucleotide sequences from regular vendors such as IDT; a standard scale (25 nmol) and purity (desalted) are sufficient.

? TROUBLESHOOTING

6 *Synthesizing the gRNA by IVT (Steps 6–14).* Dissolve the gRNA template oligonucleotide in nuclease-free water at a final concentration of 50 μM (e.g., 80 μL for a 4-nmol tube) by pipetting the water volume into the tube containing the gRNA 2–3 times, gently shaking, and centrifuging (2,000*g*, 20–23 °C, 5 s) to bring all the liquid to the bottom for collection.

▲ CRITICAL STEP Use nuclease-free reagents for all procedures that involve the manipulation of RNA.

7 To synthesize the gRNA in vitro, the two oligonucleotides are assembled as illustrated in Fig. 2a. Prepare the annealing reaction in a PCR tube by combining the following components:

Reagent	Volume (μL)
5 M NaCl	0.5
1× TE buffer	47
T7 adaptor oligonucleotide (100 μM)	0.55
gRNA template oligonucleotide (50 μM)	1
Total	49.05

8 Anneal the gRNA template and T7 adaptor oligonucleotide by performing a 5-min denaturation at 95 °C and then slowly cool the reaction to 4 °C in a PCR thermal cycler at a ramp rate of 0.04 °C/s.

■ PAUSE POINT The annealed IVT gRNA templates can be stored at −20 °C for several weeks.

9 Assemble the IVT reaction using the HiScribe T7 Quick High Yield RNA Synthesis Kit in 200-μL PCR tubes:

Reagent	Volume (μL)
Annealed gRNA template	8
HiScribe Quick master mix (10×)	10
T7 polymerase	2
Total	20

10 Incubate the reaction at 37 °C for 4 h or overnight.

11 Digest the DNA template with DNase I by adding 30 µL of water and 2 µL of DNase I to the reaction mixture. Incubate the samples at 37 °C for 15 min to allow the digestion to complete.

12 Heat-inactivate the DNase I by incubating the samples at 75 °C for 10 min.

13 Proceed with RNA cleanup using an RNA Clean & Concentrator kit, according to the manufacturer's instructions.

14 Measure the concentration of the gRNA on a microvolume spectrophotometer. The absorbance ratio A_{260}/A_{280} should be >1.8 and the absorbance ratio A_{260}/A_{230} should be >2.0.
PAUSE POINT The RNA can be used immediately or divided into aliquots and stored at −80 °C for up to several weeks.

15 *In vitro activity assays with Cas12a (Steps 15–18).* Assemble the Cas12a–gRNA complex by incubating 200 nM Cas12a with 250 nM gRNA (from Step 14) in 1× NEBuffer 2.1 at 37 °C for 10 min. Prepare 1 µL for each point of the calibration curve in the next steps. Make sure to account for technical replicates.

16 Prepare a calibration curve with the dsDNA trigger present at decreasing concentrations between 100 nM and 10 pM. The reaction mixture should also contain 1× NEBuffer 2.1 and 1 µM quenched, fluorescently labeled reporter ssDNA (hereafter referred to as 'FQ reporter'; see Table 1). Prepare a minimum of 3 µL for each point of the calibration curve.

17 Mix 1 µL of the Cas12a–gRNA complex from Step 15 with 3 µL of the trigger–FQ reporter mix from Step 16 to obtain final concentrations of 50 nM Cas12a, 62.5 nM gRNA and 750 nM FQ reporter in 1× NEBuffer 2.1.
CRITICAL STEP We routinely use MBN as a positive control for the degradation of the FQ reporter by a known ssDNA-specific endonuclease. Mix 0.8 µL of MBN with a 3.2-µL solution of 940 nM FQ reporter in 1× NEBuffer 2.1. The fluorescence signal observed in the MBN reaction should be similar to that obtained with Cas12a when using a high concentration of dsDNA trigger (50 nM).

18 Incubate 3-µL reactions (sampled from the mixtures prepared in Step 17) in a 384-well microplate and place the plate in the microplate reader. Record fluorescence readings every 5 min (excitation: 485 nm; emission: 535 nm) for 120 min at 37 °C.
? TROUBLESHOOTING

Creating CRISPR-responsive DNA hydrogels

19 For fluorophore or enzyme release from CRISPR-responsive PEG hydrogels, follow option A or B, respectively. For CRISPR-responsive polyacrylamide hydrogels encapsulating entrapped nanoparticles or cells, follow option C or D, respectively. For CRISPR-responsive µPAD diagnostics, follow option E. See 'Experimental design' section for details.

(A) **Fluorophore release from CRISPR-responsive PEG hydrogels** ● **Timing 4 d**

(i) *PEG-DNA gel synthesis (Step 19A(i–xii)).* Design and purchase Cas12a-sensitive, AT-rich (>70%) ssDNA linkers (20–40 nt) that exhibit little secondary structure. In silico tools for verifying the thermodynamic properties of interacting nucleic acid strands, such as the IDT OligoAnalyzer Tool (www.idtdna.com/oligoanalyzer), can be used to confirm the absence of secondary structure. Pick sequences such that all predicted secondary structures have a melting temperature <30 °C. To avoid unpredicted intermolecular interactions, make sure the ssDNA linkers show no complementarity to the DNA target and gRNA designed in Steps 1–5. Include a protected 5′-end thiol modification for the attachment to the PEG-VS groups (see examples in Table 1) and a 3′-chemical handle that does not react with thiols or VSs for the attachment of the cargo molecules. In the example used here, the molecular cargo (cyanine dye Cy3) was grafted onto the 3′ end of the oligos during DNA synthesis by the manufacturer.

(ii) Resuspend the pellet of oligonucleotide linkers at 500 µM in nuclease-free water. If the cargo is not pre-attached, attach the molecule of interest to the DNA using the chemistry of choice, making sure to thoroughly purify the functionalized product and adjust its molarity.
? TROUBLESHOOTING

(iii) Mix 10 µL of oligonucleotides (5 nmol) with 0.5 µL of TCEP (250 nmol). Incubate the reaction mix for 4 h at room temperature (20–23 °C) in the dark to chemically reduce the protecting groups on the ssDNA.

(iv) Resuspend an aliquot of PEG-VS powder at a concentration of 4% (wt/vol) in 1 M TEA. Make sure to adjust the volume of the PEG powder based on the density indicated by the manufacturer.

▲ **CRITICAL STEP** The TEA acts as a buffer and also as a catalyst in the subsequent Michael-type thiol–ene addition reaction with the PEG macromers. If the use of TEA is detrimental for your application, consider testing another tertiary amine catalyst or using free-radical addition chemistry.

(v) Add 62.5 µL of PEG-VS solution to the reduced oligonucleotides from Step 19A(iii) and incubate overnight (18 h) in the dark at room temperature.

▲ **CRITICAL STEP** This incubation ensures that the majority of the ssDNA is grafted onto the PEG-VS precursors. Any unreacted DNA (and attached cargos) will diffuse away from the gels during the wash step that follows the hydrogel formation (Step 19A(xii)).

■ **PAUSE POINT** The functionalized PEG-VS can be stored at 4 °C for an additional 24 h.

(vi) In the tube containing the DNA-functionalized PEG-VS, add an additional 62.5 µL of PEG-VS solution. This should be diluted in 1 M TEA to the appropriate concentration (e.g., 2% (wt/vol) to make a 1.5% PEG-DNA gel; see Box 1) from the stock prepared in Step 19A(iv).

(vii) Resuspend an aliquot of PEG-SH powder in water at the appropriate concentration (e.g., 3% (wt/vol) to make a 1.5% PEG-DNA gel; see Box 1).

? TROUBLESHOOTING

(viii) On ice, add 125 µL of the PEG-SH solution from Step 19A(vii) to the solution of functionalized PEG-VS macromers from Step 19A(vi). Adjust the volume to 500 µL with water (239.5 µL).

(ix) Dispense the hydrogel precursor solution into the final containers while it is still liquid. The polymerization time will depend on the hydrogel concentration and should take at least 5–10 min if the solution is kept cold. For the subsequent real-time monitoring of the reactions in a 96-well plate using a plate reader, tilt a non-treated, flat-bottom plate at an angle of about 45° and cast 5 µL of the precursor onto the lower edges of the wells (see Fig. 4a).

▲ **CRITICAL STEP** The kinetics of in-gel Cas12a actuation is affected by diffusion and therefore by the shape of the hydrogels. It is important to cast the gels with a consistent size and shape. This will help to maximize the reproducibility of the experiment.

? TROUBLESHOOTING

(x) Seal the wells of the 96-well plates with light- and gas-impermeable microplate sealing tape. Leave the gels in the tilted plate at room temperature overnight to allow the polymerization of the PEG macromers and the formation of the hydrogels.

(xi) Block any unreacted vinyl sulfone groups by incubating the gels for 4 h at 37 °C in a large excess of 20 mM dithiothreitol freshly dissolved in 1× NEBuffer 2.1.

(xii) Wash the gels in a large volume (at least 30-fold excess) of 1× NEBuffer 2.1 at 37 °C. Replace the buffer at least three times, incubating the gels for no less than 4 h each time, to ensure complete equilibration between the gels and the supernatant.

■ **PAUSE POINT** If the plate is properly sealed to prevent dehydration, the hydrogels can be stored in 1× NEBuffer 2.1 at 4 °C for several days. Bring the gels back to a temperature of 37 °C for several hours before use because the equilibrium swelling volume is temperature dependent.

(xiii) *Cas12a-mediated fluorophore release from PEG-DNA hydrogels (Step 19A(xiii–xvii)).* Prepare a fresh stock of gRNA-primed Cas12a enzyme (1 µM Cas12a with a two-fold molar excess of gRNA from Step 14) in 1× NEBuffer 2.1. Incubate the stock for 10 min at 37 °C to allow the enzymes and the gRNAs to form functional complexes.

(xiv) Overlay the 5-µL cargo-functionalized PEG-DNA hydrogels (from Step 19A(xii)) with 95 µL of 1× NEBuffer 2.1 pre-warmed to 37 °C.

(xv) Add 5 µL of warm Cas12a–gRNA solution from Step 19A(xiii) to the supernatant of each hydrogel to reach a final enzyme concentration of 50 nM. From this point on, incubate the gels at 37 °C on a rocker bed. For experiments monitoring the release of a fluorophore in real time in a microplate reader, set the instrument to shake the plate for 1 s every minute.

▲ **CRITICAL STEP** Include reference samples that lack Cas12a enzyme but contain unattached cargo at the theoretical concentration expected for the scenario in which 100% of it is released. For example, for a 5-µL hydrogel made from precursors containing 10 µM

of oligonucleotide-bound fluorophore, make a control overlaid with 100 µL of buffer containing 476 nM of cargo.

(xvi) Start monitoring the release of the cargo from the gels (Fig. 4b). For the real-time detection of a fluorescent reporter, pick wavelengths that maximize the signal-to-noise ratio rather than the absolute amplitude (for Cy3, excitation: 555 nm; emission: 625 nm) and minimize the frequency of the measurements to limit photobleaching. If the cargo is not suitable for in-well reaction monitoring (e.g., enzymes), sacrifice wells at each time point by sampling the supernatant and testing for activity with the appropriate assay.

? TROUBLESHOOTING

(xvii) At the desired time point, spike in 1 µL of a 5 µM stock of the dsDNA trigger (in water, Tris, or PBS) from Step 16 to reach a 1:1 enzyme-to-target molar ratio.

▲CRITICAL STEP Include appropriate controls in your experiment, namely hydrogels exposed to Cas12a–gRNA only and hydrogels exposed to non-target dsDNA fragments.

(B) **Enzyme release from CRISPR-responsive PEG hydrogels ● Timing 4 d**

(i) *PEG-DNA gel synthesis (Step 19B(i–x))*. Design and purchase Cas12a-sensitive, AT-rich (>70%) ssDNA linkers (20–40 nt) that exhibit little secondary structure. Include a 5′-end thiol modification for their attachment to the PEG-vinyl sulfone groups (see examples in Table 1) and a 3′-end biotin modification for the attachment of the enzyme. In silico tools to verify the thermodynamic properties of interacting nucleic acid strands such as the IDT OligoAnalyzer Tool (www.idtdna.com/oligoanalyzer) can be used to confirm the absence of secondary structure. Pick sequences such that all predicted secondary structures have a melting temperature <30 °C. To avoid unpredicted intermolecular interactions, make sure the ssDNA linkers show no complementarity to the DNA target and gRNA designed in Steps 1–5.

(ii) Obtain streptavidin-tagged enzymes that can be attached to the material. In the example illustrated here, we use HRP.

? TROUBLESHOOTING

(iii) Synthesize 3-µL PEG-DNA hydrogels in PCR tubes using the same method as outlined in Step 19A(ii–ix), with a final PEG concentration of 1.5% (wt/vol) and 5 µM ssDNA anchors.

(iv) After casting the gels, wash them overnight in 100 µL of 1× NEBuffer 2.1 to remove unreacted ssDNA.

(v) Make a fresh stock of 20 mM dithiothreitol in 1× NEBuffer 2.1, accounting for 200 µL of buffer per gel and a small degree of pipetting error.

(vi) Block the unreacted vinyl sulfone groups by incubating the gels with 200 µL of 20 mM dithiothreitol in 1× NEBuffer 2.1 for 4 h at 37 °C.

(vii) Rinse the gels three times in 200 µL of 1× NEBuffer 2.1, incubating for 1 h at 37 °C each time.

(viii) After the final rinse, incubate the gels for 16 h at room temperature in a 200-µL solution of 1× NEBuffer 2.1 containing 55 µg/mL of streptavidin-conjugated HRP from Step 19B(ii).

(ix) Wash the enzyme-conjugated gels through successive incubations of no less than 2 h in room-temperature 1× NEBuffer 2.1 until there is no residual HRP activity in the supernatant. To measure the HRP in the supernatant, take a 20-µL aliquot and dilute it fivefold in a TMB/E substrate solution (80 µL). Incubate the reaction for 20 min at 37 °C before checking for the appearance of the blue TMB/E oxidation product, per the manufacturer's guidelines, making sure to include negative controls. Typically, this requires 5–10 rounds of washing.

(x) Confirm the immobilization of the active enzyme in the gel by overlaying the gel with 50 µL of TMB/E substrate. The substrate should be rapidly oxidized in contact with the gel.

(xi) *Cas12a-mediated enzyme release from PEG-DNA hydrogels (Step 19B(xi–xvi))*. Prepare a reaction mix on ice in 1× NEBuffer 2.1 containing 10 nM Cas12a, 20 nM gRNA (from Step 14) and 10 nM trigger or scrambled dsDNA (Step 16, Table 1). Pre-warm the reaction mix to 37 °C immediately before the assay.

(xii) Overlay 20 µL of the reaction mix onto each 3-µL gel sample from Step 15. Incubate the gels at 37 °C on a rocker bed. The supernatant volume can be increased to 50 µL if desired.

(xiii) At the desired time points, sacrifice replicate samples by removing their supernatants and store these at 4 °C until all samples from all time points have been collected.

(xiv) Once the final time point has been reached, pipette 10 µL of each supernatant sample into 40 µL of TMB/E substrate in a 96-well plate (a fivefold dilution).

(xv) Incubate the plate at 37 °C with shaking for 10 min and then block the reaction with 50 µL of 1 M sulfuric acid.

(xvi) Measure the absorbance at 450 nm with a plate reader.

? TROUBLESHOOTING

(C) **Nanoparticle release from CRISPR-responsive polyacrylamide-DNA hydrogels** ● **Timing 3 d**

(i) *Designing and ordering the DNA components (Step 19C(i,ii))*. For the preparation of acrylamide-DNA gels, three ssDNA oligos are required: two 5′-methacryl-functionalized strands that are separately incorporated into polyacrylamide chains (designated X and Y) and a bridging 'linker' strand (designated L-15) that can be functionalized with fluorescent dyes (resulting in L-15F) (Table 1). Design each 5′-methacryl oligo X and Y to have a 5-nt TTATT sequence, followed by a 15-bp sequence that binds to the linker strand. The linker strand has two 15-bp regions that bind to X and Y, separated by a 15-bp sequence consisting of a $(TTATT)_3$ motif (Fig. 5). See Table 1 for example sequences. We outsourced the 1-µmol scale synthesis of HPLC-purified ssDNAs to IDT.

▲ **CRITICAL STEP** L-15F should be used in place of L-15 when observing the gel itself by fluorescence imaging.

? TROUBLESHOOTING

(ii) Resuspend oligos X, Y, L-15F and L-15 to a stock concentration of 3 mM in nuclease-free water and vortex them for 10 s to ensure the pellet dissolves.

■ **PAUSE POINT** The DNA stocks can be used immediately or stored at −20 °C for up to 2 months.

(iii) *Polymerizing the gel precursors (Step 19C(iii–x))*. Prepare a 10× concentration stock of hydrogel buffer (TAE–Mg^{2+}) in advance as follows:

Reagent	Volume (mL)
50× TAE buffer	20
1 M Magnesium acetate	12.5
Nuclease-free water	67.5
Total	100

(iv) For a typical preparation of the gel precursor strands X and Y, perform 180-µL reactions in 1.7-mL microcentrifuge tubes. This protocol can be scaled down to 50 µL and up to 400 µL. For 4% (wt/vol) acrylamide gels, add the reagents below in the following order. Prepare one gel precursor for the strand X and one for strand Y:

Reagent	Dilution	Final concentration	Volume (µL)
10× TAE-Mg^{2+} buffer	10×	1×	18.0
3 mM X or Y	3×	1 mM	60.0
40% (wt/vol) acrylamide	10×	4%	18.0
Nuclease-free water			75.0
		Total	171

▲ **CRITICAL STEP** For 7% (wt/vol) acrylamide gels, instead add 31.5 µL of 40% (wt/vol) acrylamide and 61.5 µL of nuclease-free water.

(v) Vortex the microcentrifuge tubes briefly and spin them down for 3 s at 2,000*g* (room temperature) in a centrifuge to collect their contents.

(vi) Place the open tubes under a vacuum for 10 min to degas the solutions. We use a vacuum desiccator chamber with the desiccant removed.

(vii) During this incubation period, prepare fresh stocks of 2% (wt/vol) APS in 1 mL of nuclease-free water and 20% (vol/vol) TEMED in 0.5 mL of nuclease-free water ('Reagent setup' section).

(viii) Add 4.5 µL of the APS stock to the polymerization reaction from Step 19C(vi), invert the tube a few times to mix the reagents, and then spin it down briefly (2,000*g*, 20–23 °C, 5 s) to collect the contents of the tube.

▲ **CRITICAL STEP** This step is time sensitive; proceed rapidly to the next step.

(ix) Add 4.5 μL of the TEMED stock to the polymerization reaction, invert the tube a few times to mix the reagents and then spin it down briefly (2,000*g*, 20–23 °C, 5 s) to collect contents of the tube.

▲ **CRITICAL STEP** This step is time sensitive; proceed rapidly to next step.

(x) Place the microcentrifuge tubes in the vacuum chamber again to allow the polymerization to proceed under vacuum for 15 min at room temperature.

■ **PAUSE POINT** The gel precursors PA-X and PA-Y can be stored in TAE–Mg^{2+} buffer at 4 °C for up to a month.

? TROUBLESHOOTING

(xi) *Crosslinking and handling macroscopic polyacrylamide-DNA gels (Step 19C(xi,xii))*. To generate the crosslinked hydrogel, mix equal volumes of the polymer solutions PA-X and PA-Y (e.g., 10 μL each) from the 'Reagent setup' section with 6 μL of the linker DNA (3 mM stock) for a final ratio of 1:1:0.6.

? TROUBLESHOOTING

(xii) After adding the linker DNA, mix the gel gently with the tip of a pipette and incubate it at room temperature for 10 min. The gelation process should begin immediately.

(xiii) *Qualitatively assessing DNA incorporation into polyacrylamide chains (Step 19C(xiii–xix))*. Prepare a 2% (wt/vol) agarose gel containing 1× NorthernMax-Gly running buffer for electrophoresis.

(xiv) As standards for the unreacted methacryl oligos (X and Y), mix 4-μL aliquots containing 10 pmol of each oligo from Step 19C(ii) with 4 μL of NorthernMax-Gly gel loading dye.

(xv) Take 2-μL samples of the polymerized precursors (4% or 7% PA-X and PA-Y, Step 19C(xii)), mix them with 2 μL of nuclease-free water and add 4 μL of NorthernMax-Gly gel loading dye.

(xvi) Heat the samples from Step 19C(xiv and xv) with NorthernMax-Gly gel loading dye in PCR tubes at 50 °C for 30 min.

(xvii) Submerge the 2% (wt/vol) agarose gel in NorthernMax-Gly gel running buffer.

(xviii) Load a low-molecular-weight DNA ladder, the unreacted oligo standards and the polymerized gel precursors into the agarose gel.

(xix) Run the gel for 1.5 h at 80 V and image the gel under UV light with a standard gel imager. If the incorporation of ssDNA into the polyacrylamide chains was successful, the lanes containing PA-X and PA-Y should appear as high-molecular-weight smears, whereas the low-molecular-weight bands of the unreacted oligonucleotides should be absent or very faint.

(xx) *Preparing PEG-functionalized gold nanoparticles (Step 19C(xx–xxvi))*. Prepare 50 mL of aqueous solution containing 0.1 mg/mL gold (III) chloride trihydrate.

▲ **CRITICAL STEP** The procedure in this section describes how to probe the release of PEG-functionalized (AuNPs from PA-DNA hydrogels. The procedure can easily be adapted to study the release of FITC-dextran. To do so, skip Step 19C(xxi–xxvi) and directly proceed to 'Preparing the Cas12a reaction master mix' in Step 19C(xxvii).

(xxi) Heat the solution and stir it vigorously on a plate heater until it boils.

(xxii) Add 1 ml of 20 mg/mL sodium citrate to the reaction while continuing to heat and stir for 15 min.

▲ **CRITICAL STEP** You should see a color change from faint yellow to deep red, indicating AuNP formation.

(xxiii) Turn off the heat and continue to stir the reaction until the solution reaches room temperature.

(xxiv) Add 0.33 mL of 2 mM 5-kDa thiol-conjugated PEG. Mix the reaction well and leave it overnight at room temperature to allow PEG conjugation to the AuNPs.

(xxv) Use a UV–Vis spectrophotometer to determine the concentration of the NPs on the basis of an extinction coefficient of 5.7×10^8 $M^{-1}cm^{-1}$ at 520 nm.

(xxvi) To wash and concentrate the AuNPs to the desired level, use a centrifuge to spin down the solution in microcentrifuge tubes at 13,000*g* for 30 min at room temperature. Remove most of the supernatant and replace it with phosphate-buffered saline or adjust the final volume to the desired concentration.

■ **PAUSE POINT** The PEG-stabilized AuNPs can be stored for months at room temperature or at 4 °C. Do not freeze solutions containing AuNPs because they will become irreversibly aggregated.

? TROUBLESHOOTING

(xxvii) *Preparing the Cas12a reaction master mix (Step 19C(xxvii))*. In a PCR tube on ice, prepare the Cas12a–gRNA master mix containing NEB Cas12a (10 μM) and gRNA from Step 14 (15 μM) in 1× NEBuffer 2.1. Each hydrogel sample being assayed will require 1 μL of the Cas12a–gRNA

master mix. Remember to account for pipetting error by preparing a slight excess of the master mix. For example, 15 µL of the Cas12a–gRNA reaction master mix is prepared by combining the following components in the order shown:

Reagent	Dilution	Final concentration	Volume (µL)
Nuclease-free water			10.87
10× NEB 2.1. buffer	10×	1×	1.50
NEB Cas12a, 100 µM	10×	10 µM	1.50
gRNA (200 µM stock)[a]	13×	15 µM	1.13
		Total	15

[a]This component is produced in Step 14 and can be adjusted to the desired concentration after the IVT process.

▲ **CRITICAL STEP** Prepare the Cas12a master mix immediately before preparing the acrylamide-DNA hydrogels for gel release experiments.

(xxviii) *Releasing AuNPs or FITC-dextran from polyacrylamide-DNA hydrogels (Step 19C (xxviii–xxxvi))*. Prepare a 24-well tissue culture plate for the AuNP release measurement according to the diagram in Fig. 6.

(xxix) Prepare the gels in volumes of 9 µL in PCR tubes at room temperature by combining the following reagents in the order shown:

Reagent	Prepared in Step	Volume (µL)
PA-X (4% or 7%)	19C(x)	3
PA-Y (4% or 7%)	19C(x)	3
10× NEBuffer 2.1	Reagent setup	0.67
PEGylated AuNPs (5 µM)	19C(xxvi)	1
Cas12a-gRNA master mix	19C(xxvii)	1
Trigger or scrambled dsDNA	Reagent setup (Table 1)	0.33
	Total	9

▲ **CRITICAL STEP** Mix the contents of the PCR tube thoroughly by pipetting up and down gently and mixing with the pipette tip. The 9-µL volume can be scaled up to create enough volume to make aliquots for multiple gels.

▲ **CRITICAL STEP** The procedure can be adapted to probe the release of FITC-dextran. To do this, replace the PEGylated AuNPs with 1 µL of 2.5 mg/mL 500-kDa MW FITC-dextran.

(xxx) Transfer 9 µL of the pre-gel mixture to the Press-to-Seal silicone isolators at the center of each well of a 24-well tissue culture plate.

(xxxi) To crosslink each gel, pipette 1 µL of the 3 mM ssDNA linker L-15 (from Step 19C(ii)) directly into the 9 µL pre-gel mix.

(xxxii) Stir the gels briefly with a pipette tip, to ensure the ssDNA linker is mixed, and incubate the plate at room temperature for 20 min.

(xxxiii) After gelation, gently add 850 µL of 1× NEBuffer 2.1 to each well and ensure the gels are fully submerged in the supernatant. If necessary, rock the plate gently by hand to cover the gels.

▲ **CRITICAL STEP** When adapting the approach to probe FITC-dextran release, incubate the tubes at 37 °C without shaking. At the desired time points (every 4 h in our case), invert the tubes once and image them using a gel imager (excitation: 385 nm; emission: 525 nm). Steps 19C(xxxiv–xxxvi) can be omitted.

? TROUBLESHOOTING

(xxxiv) Seal the plate using an optically clear plate seal, ensuring that the seal adheres across the whole plate to prevent evaporation.

(xxxv) Using a plate reader, record the release of AuNPs by measuring the absorbance at 520 nm at the center of each well of the 24-well plate (Fig. 6). Maintain the plate at a constant temperature of 37 °C and record the absorbance at 2-min intervals for at least 15 h.

(xxxvi) To complement the absorbance reading of the gel itself, readings of the supernatant can be taken at the final time point. Take a 200-µL aliquot of the supernatant from each sample and transfer it to a 96-well plate for measurement. As a standard for 100% nanoparticle release, include wells containing a 1:850 dilution of the AuNP starting stock from Step 19C (xx). Measure the absorbance at 520 nm for each sample.

? TROUBLESHOOTING

(D) **Human primary cell release from CRISPR-responsive polyacrylamide-DNA hydrogels**

● **Timing 2 d**

(i) *Pre-culturing human primary cells (Step 19D(i–v))*. First, follow Step 19C(i,ii) to design and order the DNA components.

(ii) Expand the PBMCs in R10 medium until they reach a density of ~0.5 × 10^6/mL.

(iii) Detach the PBMCs from the plate through gentle pipetting with a 10-mL serological pipette.

(iv) Centrifuge the cell suspension in a 15-mL Falcon tube at 300*g* for 5 min at room temperature to collect the cells.

(v) Resuspend the cells in R10 medium to a final concentration of 10^7 cells/mL.

(vi) *Preparing reagents for cell encapsulation (Step 19D(vi–xii))*. Follow Step 19C(iii–x) to create the PA-X and PA-Y gel precursors. Prepare pre-gel (partially crosslinked) PA-X and PA-Y stocks by mixing 10 µL of PA-X, 10 µL of PA-Y and 6 µL of 10 µM ssDNA linker (L-15).

(vii) Mix the reaction well and let it crosslink for 10 min at room temperature.

(viii) To remove any unreacted acrylamide monomer that may compromise the viability of the cells, clean the pre-gelled solution using a centrifugal spin filter (Amicon, 10-kDa, 0.5-mL spin filter). Wash the pre-gel sample with 0.45 mL of 1× PBS and spin it down at 14,000*g* for 15 min at room temperature.

(ix) Repeat the washing and filtering process (Step 19D(viii)) two additional times.

(x) Measure the concentration of ssDNA in the pre-gels with a microvolume spectrophotometer. Dilute the sample in 1× PBS to reach a final ssDNA concentration of 1.8 mg/mL. In the upcoming steps, we will refer to this mixture as 'PA-XYb'.

(xi) Filter a 2 M magnesium chloride stock solution with a 0.22-µm membrane (e.g., Steri-Flip or syringe filter) inside a laminar flow hood while maintaining standard sterile technique.

(xii) Prepare a sterile working stock of 200 mM magnesium chloride in the laminar flow hood.

(xiii) *Generation of cell-encapsulating PA-DNA hydrogels (Step 19D(xiii–xviii))*. Prepare a 30-µL aliquot of a suspension of ssDNA linker, magnesium chloride and human cells by combining the following reagents in the order listed:

Reagent	Volume (µL)
ssDNA linker L-15 (3 mM)	3.6
Fluorescent ssDNA linker L-15F (3 mM)	0.4
Cells at 10^7 cells/mL (from Step 19D(v))	8
Magnesium chloride (200 mM) (from Step 19D(xii))	6
10× PBS	3
Nuclease-free water	9
Total	30

▲ **CRITICAL STEP** An intercalating DNA gel stain (e.g., EvaGreen) can be used instead of a fluorescent ssDNA linker L-15F.

(xiv) Prepare a 30-µL aliquot of clean PA-XYb pre-gel mixture (from Step 19D(ix)).

(xv) In a black, clear-bottom 96-well plate, fill the wells immediately surrounding the well being used for the experiment with water. This helps to reduce evaporation from the gel during the upcoming incubation periods.

(xvi) Just before depositing the gels, thoroughly mix 30 µL of the filtered PA-XYb pre-gel (from Step 19D(xiv)) with 30 µL of the ssDNA linker–magnesium chloride–cell suspension (from Step 19D(xiii)) until the gel is homogeneous and the flow is highly viscous.

(xvii) Deposit 2-µL droplets of the suspension containing human cells and the various gel components at the centers of the wells of a 96-well plate.

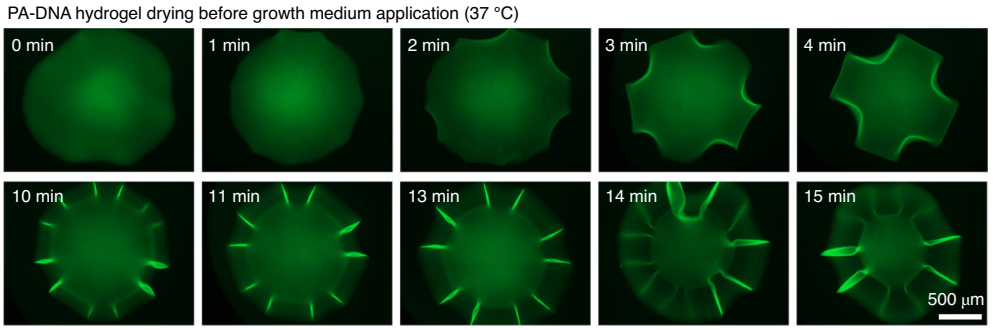


Fig. 13 | Time-dependent drying of PA-DNA hydrogels. Structural alterations caused by hydrogel drying after 2 min define this as the optimal incubation time for gel crosslinking before overlaying the supernatant (cell growth medium).

▲ **CRITICAL STEP** The black, glass-bottom 96-well plate being used must be sterile and must not be tissue culture treated. Tissue culture–treated plates have a hydrophilic surface treatment that causes the gel droplets to spread.

(xviii) Incubate the plates at 37 °C in a tissue culture incubator for 1–15 min (2 min is ideal; see Fig. 13) to allow the gels to crosslink.

? TROUBLESHOOTING

(xix) *Depositing medium on top of cell-loaded PA-DNA hydrogels (Step 19D(xix,xx))*. Add 100 µL of pre-warmed (37 °C) O10 medium (Reagent setup) to the hydrogel and incubate for 5 min at 37 °C.

▲ **CRITICAL STEP** Mix the growth medium and deposit it into each well from the side, making sure not to dislodge the gel itself.

(xx) Observe the gels under a microscope to assess their integrity before any further testing. Gels should be homogeneous, with no signs of mechanical disruption.

(xxi) *Releasing cells from PA-DNA hydrogels (Step 19D(xxi–xxiii))*. Make a 26-µL aliquot of the Cas12a–gRNA master mix by combining the reagents below and then add this mixture gently to the well containing the PA-DNA hydrogel and cell culture media.

Reagent	Volume (µL)
Cas12a (100 µM)	2
gRNA from Step 14	2
10× NEBuffer 2.1	2
Trigger DNA (100 µM) from Step 17	20
Total	26

▲ **CRITICAL STEP** Negative controls can be generated by substituting the trigger DNA with scrambled DNA. The concentration of the trigger dsDNA can be modified.

▲ **CRITICAL STEP** Avoid the mechanical disruption of the cell-encapsulating hydrogels by minimizing fluid shear and excessive movement.

(xxii) Record the fluorescence of the gels (excitation: 490 nm; emission: 535 nm) on an inverted fluorescence microscope to measure their degradation at 37 °C. We recommend imaging the gels every hour to assess their degradation kinetics.

(xxiii) Image the morphology of the PA-DNA hydrogels once the desired period of Cas12a degradation at 37 °C has elapsed. Acquire the images using a 4× magnification objective, 30% LED illumination intensity and 50% contrast. Compare these images with images of the initial state of the gels at the beginning of the experiment.

(xxiv) *Assessing the viability of cells released from PA-DNA hydrogels (Step 19D(xxiv,xxv))*. Once the gel degradation experiment is complete, assess the viability of the cells by staining with ethidium homodimer-1 and Calcein Blue, AM, by following the manufacturer's instructions.

(xxv) Observe viable PBMCs released from the hydrogels using the digital inverted microscope in DAPI mode (excitation: 360 nm; emission: 447 nm) with a 20× objective, 40% LED illumination intensity and 90% contrast. Dead cells can also be imaged using the RFP mode (excitation: 530 nm; emission: 593 nm) with 50% LED illumination intensity and 90% contrast.

(E) **CRISPR-mediated μPAD diagnostics (2 d)** ● **Timing 2 d**

(i) *Fabricating the μPAD base construct (Step 19E(i–xi))*. First, follow Step 19C(i,ii) to design and order the DNA components.

(ii) Prepare PA-X and PA-Y gel precursors as described in Step 19C(iii–x).

(iii) Align a sheet of chromatographic filter paper on the Xerox Phaser 8560 wax printer.

(iv) Print side A of the provided pattern (Supplementary Data 1).

(v) Turn the printed paper and print side B of the provided pattern (Supplementary Data 2), ensuring that the printed design is aligned on both sides of the paper.
▲ **CRITICAL STEP** Validate μPAD alignment from both sides of the paper by checking against a light source.
? TROUBLESHOOTING

(vi) Wax-reflow the printed μPADs to ensure wax penetration through the paper fibers by pressing each printed sheet for 15 s at 125 °C with a hot press.
▲ **CRITICAL STEP** Different hot press equipment and substrates will require the optimization of reflow conditions to achieve similar results.

(vii) Allow the μPADs to cool to room temperature.

(viii) Cut and fold the individual μPADs as shown (Fig. 10) so that the layers overlap like an accordion when pressed together.
▲ **CRITICAL STEP** Ensure that layers 1 to 4 of the μPADs exhibit circular hydrophilic paper regions of ~1.5 mm in diameter, surrounded by an evenly distributed hydrophobic wax coating to prevent undesired lateral flow. Layer 5 of the μPADs contains a 1.5 × 30-mm lateral flow channel with marked lengths.
? TROUBLESHOOTING

(ix) Extend the μPAD reaction layers and fill layer 3 with 0.5 μL of a mixture containing PA-X and PA-Y (1:1 (vol/vol)).

(x) Fill layer 4 with 0.5 μL of 1× PBS containing food color dye diluted 5× in PBS.

(xi) Freeze-dry the paper μPADs with the deposited reagents for 4–8 h, using a lyophilizer.
■ **PAUSE POINT** The lyophilized μPADs can be stored for several days in airtight bags with desiccant pouches.

(xii) *Preliminary μPAD operation tests (Step 19E(xii–xiv))*. Test the basic operation of freeze-dried μPADs by adding 0.3 μL of either 1 mM or 100 μM ssDNA linker (L-15) to layer 2. Next, fold all of the layers together and deposit 10 μL of PBS on top of layer 1. The folded layers can be held together using a standard paperclip or other clamp. In the case in which 1 mM L-15 is used, there should be no flow in the lateral channel because of crosslinking of the PA gel, which will impede capillary flow. Similarly, when using 100 μM L-15, minimal flow should be observed in the lateral channel. This method can be used to validate the effective formation of gels in the μPAD at high concentrations of the ssDNA linker.
▲ **CRITICAL STEP** Visible flow beyond 5 mm on the lateral channel indicates ineffective gelation or defects in the fabrication of the μPAD.
? TROUBLESHOOTING

(xiii) Once both the processes of flow and gel polymerization have been validated in the μPAD, colorimetric tests can be conducted. To layer 2 of the μPAD, add 0.3 μL of a 100 μM solution of ssDNA linker (Reagent setup and Table 1) that has been pre-incubated for 4 h at 37 °C with 300 nM Cas12a, 1 μM gRNA (from Step 7) and dsDNA trigger (Reagent setup and Table 1) in 1× NEBuffer 2.1. To assess the sensitivity of the device, use varying concentrations of the dsDNA trigger (e.g., 0 nM, 0.4 nM, 2 nM, 10 nM and 50 nM).

(xiv) Assess the degree of cleavage of the ssDNA bridge by Cas12a by measuring the length of flow of the buffer through the lateral channel of the μPAD at an endpoint time of 5 min. The distance traveled by the dye-containing buffer should be correlated with the initial concentration of the dsDNA trigger in the pre-incubation reaction.
▲ **CRITICAL STEP** The μPAD design can be modified to generate analog and wireless digital electronic readouts. To do so, follow the instructions presented in Box 2 and Box 3, respectively. Then proceed to the amplification and detection steps (Step 19E(xv–xxiii)).

Box 2 | Modification of the μPAD for conductivity measurements ● Timing 2 h

The μPAD can be adapted for conductivity measurements. To do so, follow the instructions below after completing Step 19E(iv):

1 Cover layer 5 of the μPAD with 3 × 30-mm strips of conductive tape, placed along the top and bottom sides of the lateral flow channel. These act as parallel conductive planes for the measurement of electrical resistance across the channel, as a function of the buffer wicking distance.
2 Laminate both the conductive tape and wiring over layer 5 to ensure robust electrical connection between the components. Avoid covering the fluid inlet in layer 1. To laminate the device, use clear adhesive tape cut to exceed the perimeter of the μPAD, enabling the top and bottom layers of the adhesive tape to come into contact with each other around its perimeter. This provides a seal that prevents detachment of the electrical contacts while maintaining a fluidic connection between the μPAD layers. At this stage, leave layers 1 and 2 of the μPAD uncovered to receive the conductive buffer and ssDNA linker during testing.
3 At the time of testing, fill layer 2 of the μPAD with 0.3 μL of the Cas12a–gRNA assay reaction. The Cas12a–gRNA assay mix should contain 100 μM ssDNA linker (from Step 19C(ii)), 300 nM Cas12a, 1 μM gRNA (from Step 14) and dsDNA ('Reagent setup', Table 1) at varying concentrations (e.g., 0 nM, 1 nM, 10 nM, 100 nM) in 1× NEBuffer 2.1. This reaction should be prepared separately and incubated for 4 h at 37 °C before testing with the μPAD. Negative-control reactions can be performed with scrambled dsDNA.
4 After the pre-digested ssDNA linker (L-15) solution has been deposited and air-dried for 1 min, fold the μPAD layers to fluidically connect all the hydrophilic regions. In this state, layer 1 acts as a protective cover.
5 Add 2 μL of 1× PBS to layer 1 and measure the conductivity across the channel.
6 Obtain electronic measurements from the μPAD by monitoring channel resistance with a digital multimeter. The 5-min endpoint resistance values for experiments with different concentrations of dsDNA trigger can be collated to delineate the titration curve.
7 Continue with the main procedure at Step 19E(xv).

Box 3 | RFID integration into the CRISPR-mediated stop-flow μPAD ● Timing 2 h

The μPAD can be adapted to wirelessly transmit diagnostic data through an RFID tag. To do so, follow the instructions below after completing Step 19E(iv).

1 Acquire commercial RFID tags with an exposed printed antenna (Fig. 12).
2 Inspect the RFID tags to confirm that they function as expected.
3 Print the flexible, interdigitated electrodes (Supplementary Data 3) on a polyethylene terephthalate substrate, using silver nanoparticle ink. Deposit the ink with a modified printer with refillable cartridges according to the protocol developed by Lee et al.[45].
4 Cut each printed interdigitated electrode into 20 × 20-mm squares as outlined in Supplementary Data 3.
5 Place the IDE on top of the conductive side of the UHF-RFID tag, with both conductive IDE terminals coplanar and in close proximity to the first conductive antenna loop (<10 mm away from the UHF-RFID chip). Double-sided tape can be used to prevent movement of the IDE and UHF-RFID tag (Fig. 12).
6 Connect the IDE terminals to the UHF-RFID antenna loop with two 2 × 4-mm strips of conductive tape.
7 Verify electrical conductivity between each of the IDE terminals and each side of the UHF-RFID chip with a multimeter.
? TROUBLESHOOTING
8 Cut the lateral flow channel of a multi-layer μPAD stack to a length of 20 mm and place it on top of the interdigitated electrode/UHF-RFID tag arrangement.
9 Align the bottom of the lateral hydrophilic region in layer 5 to be in direct contact with the conductive side of the IDE.
10 Test the reagent placement and activation of the μPAD by performing conductivity readings across the flow channel as described in Step 19E (xxiv–xxix).
11 Measure the relative received signal strength indicator (RSSI) of each μPAD RFID tag in the presence or absence of the target DNA. Place two RFID tags (one modified to incorporate the μPAD and one unmodified) at a distance of 0.5 m from a UHF-RFID antenna. Connect the UHF-RFID antenna to a simultaneous RFID tag reader and an Arduino microcontroller using the M6E-NANO RFID Arduino library. The Arduino code used to record this signal can be downloaded as Supplementary Software 1. A measurable decrease in the absolute RSSI values during the test indicates that the power level of the received radio signal has decreased due to the flow of conductive buffer through the device and the resulting short-circuiting of the RFID tag antenna.
12 Continue with the main procedure at Step 19E(xv).

(xv) *RT-RPA CRISPR–μPAD tests for EBOV ssRNA (Step 19E(xv–xxiii))*. Obtain a dsDNA fragment coding for the VP30 protein of the Zaire strain of the Ebola virus (ZEBOV) from a commercial manufacturer; for example, order the sequence as a gblock from Integrated DNA Technologies.
▲ CRITICAL STEP The RT-RPA section (Step 19E(xv–xx)) is required only for ssRNA targets such as the Ebola virus. When the approach is used to detect a dsDNA target, Step 19E(xv–xx) can be omitted.

(xvi) Amplify the fragments by PCR and perform IVT using a HiScribe T7 Quick High Yield RNA Synthesis Kit to produce synthetic ssRNA (see Table 1 for the relevant primers).

(xvii) Purify the transcript by using an RNA Clean & Concentrator Kit and following the manufacturer's instructions.

(xviii) Dilute the RNA by serial dilution. We recommend starting with a range of concentrations in the femtomolar to attomolar range.

(xix) Perform RT reactions on the diluted RNA samples using a Superscript IV Reverse Transcriptase kit. Follow the manufacturer's instructions for the kit and include the EBOV RPA-2 reverse primer (Table 1) in the reaction.

(xx) Add 5 µL of the reverse-transcribed RNA to lyophilized 50-µL RPA Basic reactions (from a TwistAmp Basic kit). Each reaction should contain 480 µM of each RPA primer (see Table 1) and 14 mM magnesium acetate. Perform the RPA reactions per the manufacturer's instructions.

(xxi) Incubate the RPA reactions for 40 min at 37 °C. Once the amplification is complete, add the ssDNA linker L-15 (from Step 19C(ii)), gRNA (from Step 14) and Cas12a to final concentrations of 300 µM, 0.9 µM and 0.5 µM, respectively.

(xxii) Incubate the samples for an additional 4 h and then dilute the reactions 1:1 (vol/vol) with nuclease-free water. We refer to the product of this step as the 'RT-RPA–linker mix'.

(xxiii) Test the µPADs by depositing 0.3 µL of the RT-RPA–linker mix into layer 2. The µPAD should also contain 0.3 µL of 4% (vol/vol) PA-X and PA-Y in layer 3 and 0.3 µL of 1× PBS with red dye in layer 4. Once the reagents have been added to layers 2–4, they should be air-dried for 2 min. To maintain contact between the hydrophilic sections of the device, ensure that the µPAD is fully collapsed throughout the remainder of the experiment by using a paperclip as a clamp. Finally, add 2 µL of PBS to layer 1 (the top) of the µPAD to initiate either colorimetric or electrical readings.

? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
2	A low affinity of Cas12a for its target when designing new gRNA sequences	The choice of direct repeat sequence can affect target binding performance, as has been discussed in genome editing contexts[47,48]	Check that the gRNA contains a direct repeat sequence that results in high binding performance
5	IVT yield is lower than expected	T7 transcription can be affected by the bases that immediately follow the promoter sequence	If the yield of the transcript is low, two G bases or a GCG motif can be tested instead of the three G bases at the end of the T7 RNA polymerase promoter. This should not affect downstream Cas12a activity
18	The negative control has a high background	The gRNA may be contaminated with DNase I that was neither inactivated nor washed out in the Zymo spin column	Repeat IVT (Steps 6–14) to produce fresh gRNA
	High concentrations of trigger do not lead to high fluorescence signals, as compared with a positive control with MBN	The gRNA might be contaminated with RNases	Repeat IVT (Steps 6–14) to produce fresh gRNA. If the problem persists, try selecting a new dsDNA target; it may be a target-specific issue
	Reactions performed with MBN show fluorescence, but there is no fluorescence in those with Cas12a	The gRNA or Cas12a might be degraded	Check that the gRNA sequence includes the direct repeat sequence motif and the target contains a PAM site (TTTV). Make a fresh stock of Cas12a–gRNA master mix, keeping it on ice and using it within 15 min of preparation. Do not freeze and reuse aqueous dilutions containing Cas12a
19A(ii)	The reporter molecules do not leave the gel after Cas12a activation	The cargo presents exposed chemical moieties that may have reacted with the PEG precursors (e.g., surface cysteine residues of proteins may react with vinyl sulfones)	If the cargo cannot be modified to avoid cross-reactivity, selectively graft the cargo onto the DNA in situ after forming and blocking the PEG gels (e.g., by cycloaddition on 3′-azide-modified DNA anchors, or as described for HRP)
19A(vii)	The gel polymerizes very slowly	The PEG-SH macromers are oxidized	Use freshly made PEG-SH stocks
19A(ix)	The gels are too dense to pipette	The gels polymerized in the tube before they could be added to the microtiter plate wells	Prepare fresh gels. Keep them on ice while transferring them to the microtiter plate

Table continued

Table 3 (continued)

Step	Problem	Possible reason	Solution
19A(xvi)	The degradation behavior of the gel is highly variable across technical replicates	Gel was deposited with inconsistent pipetting, which results in irregular gel shapes with different surface areas exposed to the degradation bulk	Deposit your gels on the distal side of the wells, in the columns furthest from the center of the 96-well plate. Cover and spin the plate slowly (e.g., 500*g, 20 °C, 1 h*) during the polymerization to flatten the gels and homogenize their shapes
	The degradation behavior of the gel is slower or faster than expected	The use of containers with different material and geometries than the ones specified may lead to differences in gel shape and exposed surface areas exposed to the bulk, which can affect the expected Cas12a diffusion and DNA degradation profiles	Ensure the use of the specified materials, wells and tubes in all experiments
	Some of the gels exhibit a high baseline release of cargo in the absence of trigger	The gels were cast too close to the center of the wells, where the plate reader reads the optical signal. The instrument is therefore reading hydrogel fluorescence rather than signal from the solution	Define custom plate dimensions in the plate reader software to ensure that the instrument reads fluorescence near the edge of the well, opposite to the hydrogels (Fig. 4b)
19B(ii)	There are issues with the enzymatic activity when testing enzymes other than HRP	If you are extending this protocol to test the release of other functional enzymes, then these will also need to be modified for DNA attachment. This may partially alter their functionality	Confirm that the attachment chemistry does not affect enzyme function
19B(xvi)	There is no signal in the supernatant after incubating with Cas12a	The ssDNA linkers were not cut	Perform Steps 15–18 to confirm the activity of Cas12a (i.e., that the gRNA and Cas12a are not degraded)
19C(i)	The gel polymer strands are too viscous to handle	The sequences and length of the complementary regions (15 bp in our case) can be tuned according to the user's needs. However, we attempted experiments with an 18-bp overlap for X and Y, and the resulting gel polymer strands were too viscous to handle effectively	Re-design the sequences and lengths of the complementary regions. Polymerized gel precursors can be heated to 37 °C or even up to 70 °C for short periods to help reduce viscosity while pipetting; however, avoid repetitive heating and cooling of the same stock of PS-X or PS-Y
19C(x)	There is no visible polymerization of the acrylamide	Although the polymerization does not lead to optical changes, it is possible to assess it by changes in viscosity or by comparing the sizes of DNA-stained bands on an agarose gel	To check that polymerization has occurred, use a 10-μL pipette tip to check for an increase in the viscosity of the reaction by pipetting up and down. Alternatively, run a 1-μL aliquot of the reaction on an agarose gel and look for a very slow-moving band
	The polymerization is not successful	APS and TEMED are sensitive to moisture and air	Buy new APS and TEMED vials. These chemicals are sensitive to both moisture and air and should be stored at 4 °C with a desiccant. Over time, the solutions become inactive. Degas the water you use to make the APS and TEMED stocks. Oxygen can also be removed by bubbling an inert gas through the water for some time
19C(xi)	Precursor strands are too viscous to pipette accurately	The precursor strands can be highly viscous after storage at 4 °C, particularly in the case of the 7% acrylamide gels	To ensure accurate pipetting, warm the stocks of PA-X and PA-Y to 50–70 °C in a heating block before handling. If the mixture is still too viscous to pipette, a sharp razor can be used to remove the tip of the disposable pipette tip, which increases the diameter of the opening
19C(xxvi)	The nanoparticles change color to blue/gray or black	A change in color from the characteristic red/purple of well-dispersed AuNPs to gray/black indicates aggregation	Repeat the synthesis of the AuNPs. Ensure that the glassware and the stirrer bar are cleaned thoroughly with ultrapure water before synthesizing AuNPs
19C(xxxiii)	The activated Cas12a does not cleave ssDNA	See advice for Step 18	
	The level of background leakage is unexpectedly high	Background leakage can occur due to the mesh size of the hydrogel and the mechanism of AuNP entrapment	Modify the particle size to reduce diffusion out of the gel. Possible strategies include PEGylation, attachment to the matrix, or the alteration of the properties of the gel to make it stiffer

Table continued

Table 3 (continued)

Step	Problem	Possible reason	Solution
19C(xxxvi)	The degradation behavior of the gel is slower or faster than expected	The use of containers with different material and geometries than the ones specified may lead to differences in gel shape and exposed surface areas exposed to the bulk, which can affect the expected Cas12a diffusion and DNA degradation profiles	Ensure the use of the specified materials, wells and tubes in all experiments
19D(xviii)	The gels have heterogeneous morphologies	Different crosslinking times at this stage can affect the shape and final size of the hydrogel drop due to the combined effects of bulk crosslinking and evaporation	Control the drying time of the gels (Fig. 13)
19E(v)	There is no flow in the μPAD after printing	Misalignments between the layers of as little as 1 mm can cause unreliable flow in the μPADs	The front and back of the chromatographic paper wax patterns should be perfectly aligned
19E(viii)	There is no flow in the μPAD after printing	The degree of wax reflow might have been excessive	Repeat the printing and reflow the wax at a lower temperature for longer
	There is flow outside of the wax channel	The wax reflow was ineffective	Repeat the reflow at higher temperatures for longer to ensure that the wax penetrates pores from both sides of the chromatographic paper.
	There is no flow in the μPAD due to poor alignment	The hydrophilic regions are poorly aligned	Confirm the alignment of the hydrophilic sections within 0.5 mm using a light source and visual inspection. Discard any μPADs with visible printing, reflowing or alignment defects. Ensure that layers 1–4 of the μPADs exhibit circular hydrophilic paper regions ~1.5 mm in diameter, surrounded by an evenly distributed hydrophobic wax coating to prevent undesired lateral flow
19E(xii)	There is ineffective gelation in the channels or no flow through the μPAD	The gel reagents should be tested separately in a PCR tube to confirm that the precursors crosslink rapidly when combined with the ssDNA bridge	Successful precursor crosslinking can be determined by visually inspecting the gel flow in a pipette. This should be visibly more viscous 2–5 s after the mixing of the reagents. If the gel components polymerize as described, defects in the μPAD pattern alignment or insufficient wax reflow are the most likely reasons for the device failure (see advice for Step 19E(viii))
19E(xxiii)	There is ineffective gelation in the channels or too much flow through the μPAD	The use of different equipment to print and reflow the wax might cause differences in pore and channel sizes	Ensure the μPAD channel size and geometry do not differ due to changes in printing equipment. Apply the specified volume of PBS to characterize the flow in the channel
Box 3, step 7	The DEFINE IDE is not connected to the UHF-RFID	There may be issues with the electrical conductivity of the components or with the attachment of the insulating materials	If the IDEs are not electrically connected to the UHF-RFID chip, try pressing the tape and ensuring that both the tape and the conductive silver film in the UHF-RFID are clean and free of insulating materials

Timing

Reagent setup: 3 h
Steps 1–5, design of gRNA and DNA components: 30 min each
Steps 6–14, IVT and preparation of DNA targets: 6–12 h
Steps 15–18, in vitro validation of gRNAs: 3 h
Step 19A(i–xii), PEG gel synthesis: 2–3 d
Step 19A(xiii–xvii), release of cargos: 30 min (setup) + 12 h
Step 19B(i–x), PEG-DNA gel synthesis: 2–3d
Step 19B(xi–xvi), Cas12a-mediated enzyme release from PEG-DNA hydrogels: 30 min (setup) + 6 h
Step 19C(i–xix), polymerization and synthesis validation: 3 h
Step 19C(xx–xxvi), synthesis of PEGylated AuNPs: 1 d
Step 19C(xxvii), preparing the Cas12a reaction master mix: 30 min (setup)

Step 19C(xxviii–xxxvi), releasing AuNPs or FITC-dextran from PA-DNA hydrogels: 30 min (setup) + 18 h
Step 19D(i–v), harvesting cells from cultures: 30 min
Step 19D(vi–xii), preparation of precursors for cell experiments: 3 h (synthesis) + 2 h (washing)
Step 19D(xiii–xx), embedding of cells in hydrogels: 3 h
Step 19D(xxi–xxiii), monitoring of cell release: 8 h
Step 19D(xxiv,xxv), post-release viability assay: 1 h
Step 19E(i–xiv), fabrication and testing of base μPAD: 6 h
Step 19E(xv–xxiii), RT-RPA and μPAD detection: 2 h
Box 2, modification of μPAD for conductivity measurements: 2 h
Box 3, RFID integration into the CRISPR-mediated stop-flow μPAD: 2 h

Anticipated results

On the basis of the experimental results described in our previous work[15] and presented in Fig. 14, we expect this protocol to lead to the following key observations.

For any gRNA–dsDNA pair, we expect the rate of collateral cleavage of ssDNA upon binding the trigger to depend on the dsDNA concentration, within the limits of stoichiometric Cas12a saturation (Fig. 14a)[15,22]. It is important to validate the gRNA sequence for the chosen dsDNA target because the relative performance of gRNAs and the ability of a dsDNA sequence to activate Cas12a are



Fig. 14 | Anticipated Cas12a activation by dsDNA triggers and subsequent material actuation. a, The rate of collateral ssDNA cleavage correlates with the amount of activated Cas12a and therefore the amount of gRNA-matching dsDNA input, both in solution and in hydrogels. We monitored the presence of Cy3 in the supernatant of 2% PEG hydrogels upon exposure to either a *mecA* dsDNA trigger or a scrambled control (Step 19A). Traces indicate mean ± s.d. for four technical replicates. **b**, The Cas12a–gRNA complex is inactive until it encounters a specific trigger dsDNA, after which it penetrates the PEG hydrogels and cuts ssDNA linkers. Material properties affect the kinetics of Cas12a-mediated dsDNA-specific PEG hydrogel actuation. Results were obtained as in **a**, with 50 nM of Cas12a–gRNA complex and 10 nM of dsDNA. **c**, When all components are pre-loaded into the PA-DNA hydrogel, sequence-specific Cas12a–gRNA complex activation results in a sudden disruption of the material and the release of its components into solution. We measured the absorbance through 7% PA-DNA hydrogels loaded with AuNPs, Cas12a–gRNA and dsDNA (Step 19C). Traces indicate mean ± s.d. for four technical replicates. **d**, Top, digestion of ssDNA bridges by activated Cas12a prevents gel formation in the paper-based fluidic device, resulting in a dsDNA trigger–dependent lateral flow detectable by visual and resistive readouts. Bottom, detection of buffer flow by short-circuiting an RFID antenna results in a wirelessly detectable digital signal. Panel shows two representative signals recorded after RT-RPA and μPAD testing of samples containing either 0 aM or 11 aM synthetic Ebola ssRNA (Step 19E). Adapted from English, M. A. et al. Programmable CRISPR-responsive smart materials. *Science* 365, 780–785 (2019). Reprinted with permission from AAAS.

quantitatively variable. When actuating hydrogels similar to those described here (on the millimeter scale), response times are typically on the order of hours[15]. In new material contexts, response times might differ from those presented here, which may require adjustments to the frequency of measurements to best capture the kinetics of actuation.

In both PEG-DNA and PA-DNA hydrogels, cargos trapped in the hydrogels should be released upon activation of Cas12a by the gRNA-defined dsDNA trigger but not by non-matching sequences (Fig. 14b,c)[15]. When Cas12a acts as a sentinel and the dsDNA is added later, we observed that pre-exposure to non-trigger DNA did not noticeably compromise Cas12a activation by dsDNA triggers at later time points[15].

Cas12a is a large molecule and its movement can be partially slowed by the chains of the hydrogels. As a consequence, the kinetics of ssDNA cleavage is also affected by the physical properties of the DNA-containing material in cases in which the diffusion of the enzyme through the gel is partially limiting (Fig. 14b; ref. [15]). We also observed a reduction in the background release of nanoparticles (Step 19C) when using 7% PA-DNA gels, as compared with 4% gels, consistent with an improvement in the retention of physically enmeshed cargos[15].

Despite some basal ssDNA cleavage activity of serum-rich media (Fig. 8), we observed that PA-DNA hydrogels were stable for at least several hours in the absence of Cas12a activation and released encapsulated cells (Step 19D) only in the presence of a dsDNA trigger. The encapsulation and release of the cells were not cytotoxic because even sensitive primary cells were 80–90% viable after Cas12a-mediated release[15].

In μPAD-based diagnostics (Step 19E), both the visual and analog resistive modes provided continuous readouts dependent on the wicking length of the buffer inside the lateral flow channels (Fig. 14d, top), whereas the short-circuiting of the RFID antenna typically produced sharp signal transitions and binary behaviors within minutes (Fig. 14d, bottom). For the gRNA–dsDNA pairs that we used, the limit of detection of the hydrogel-controlled μPAD diagnostic after an RT-RPA was comparable to those of other CRISPR-based technologies, with a limit of detection of ~11 aM. Without pre-amplification, the μPAD routinely detected target sequences down to 400 pM[15].

Reporting Summary

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

Data availability

The data generated or analyzed during this study are included in this article and our previous publication[15]. The original data files can be obtained from the corresponding author upon reasonable request.

References

1. Lu, Y., Aimetti, A. A., Langer, R. & Gu, Z. Bioresponsive materials. *Nat. Rev. Mater* **2**, 16075 (2017).
2. Li, J. Y. & Mooney, D. J. Designing hydrogels for controlled drug delivery. *Nat. Rev. Mater* **1**, 16071 (2016).
3. Kanamala, M., Wilson, W. R., Yang, M. M., Palmer, B. D. & Wu, Z. M. Mechanisms and biomaterials in pH-responsive tumour targeted drug delivery: a review. *Biomaterials* **85**, 152–167 (2016).
4. Wilson, D. S. et al. Orally delivered thioketal nanoparticles loaded with TNF-alpha-siRNA target inflammation and inhibit gene expression in the intestines. *Nat. Mater.* **9**, 923–928 (2010).
5. Place, E. S., Evans, N. D. & Stevens, M. M. Complexity in biomaterials for tissue engineering. *Nat. Mater.* **8**, 457–470 (2009).
6. Mura, S., Nicolas, J. & Couvreur, P. Stimuli-responsive nanocarriers for drug delivery. *Nat. Mater.* **12**, 991–1003 (2013).
7. Baker, S., Thomson, N., Weill, F. X. & Holt, K. E. Genomic insights into the emergence and spread of antimicrobial-resistant bacterial pathogens. *Science* **360**, 733–738 (2018).
8. Ji, J. F. et al. MicroRNA expression, survival, and response to interferon in liver cancer. *N. Engl. J. Med* **361**, 1437–1447 (2009).
9. Pardee, K. et al. Rapid, low-cost detection of Zika virus using programmable biomolecular components. *Cell* **165**, 1255–1266 (2016).
10. Takahashi, M. K. et al. A low-cost paper-based synthetic biology platform for analyzing gut microbiota and host biomarkers. *Nat. Commun.* **9**, 3347 (2018).
11. Gootenberg, J. S. et al. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science* **360**, 439–444 (2018).
12. Na, W., Nam, D., Lee, H. & Shin, S. Rapid molecular diagnosis of infectious viruses in microfluidics using DNA hydrogel formation. *Biosens. Bioelectron* **108**, 9–13 (2018).

13. Venkatesh, S., Wower, J. & Byrne, M. E. Nucleic acid therapeutic carriers with on-demand triggered release. *Bioconjugate Chem.* **20**, 1773–1782 (2009).
14. Li, J. et al. Functional nucleic acid-based hydrogels for bioanalytical and biomedical applications. *Chem. Soc. Rev.* **45**, 1410–1431 (2016).
15. English, M. A. et al. Programmable CRISPR-responsive smart materials. *Science* **365**, 780–785 (2019).
16. Knott, G. J. & Doudna, J. A. CRISPR-Cas guides the future of genetic engineering. *Science* **361**, 866–869 (2018).
17. Jiang, W. Y. & Marraffini, L. A. CRISPR-Cas: new tools for genetic manipulations from bacterial immunity systems. *Annu. Rev.Microbiol.* **69**, 209–228 (2015).
18. Gootenberg, J. S. et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* **356**, 438–442 (2017).
19. Myhrvold, C. et al. Field-deployable viral diagnostics using CRISPR-Cas13. *Science* **360**, 444–448 (2018).
20. Kellner, M. J., Koob, J. G., Gootenberg, J. S., Abudayyeh, O. O. & Zhang, F. SHERLOCK: nucleic acid detection with CRISPR nucleases. *Nat. Protoc.* **14**, 2986–3012 (2019).
21. Li, S. Y. et al. CRISPR-Cas12a-assisted nucleic acid detection. *Cell Discov.* **4**, 20 (2018).
22. Chen, J. S. et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science* **360**, 436–439 (2018).
23. Li, S. Y. et al. CRISPR-Cas12a has both *cis*- and *trans*-cleavage activities on single-stranded DNA. *Cell Res.* **28**, 491–493 (2018).
24. Singh, D. et al. Real-time observation of DNA target interrogation and product release by the RNA-guided endonuclease CRISPR Cpf1 (Cas12a). *Proc. Natl. Acad. Sci. USA* **115**, 5444–5449 (2018).
25. Strohkendl, I., Saifuddin, F. A., Rybarski, J. R., Finkelstein, I. J. & Russell, R. Kinetic basis for DNA target specificity of CRISPR-Cas12a. *Mol. Cell* **71**, 816–824 (2018).
26. Carlson, R. The changing economics of DNA synthesis. *Nat. Biotechnol.* **27**, 1091–1094 (2009).
27. Gao, M., Gawel, K. & Stokke, B. T. Toehold of dsDNA exchange affects the hydrogel swelling kinetics of a polymer-dsDNA hybrid hydrogel. *Soft Matter* **7**, 1741–1746 (2011).
28. Sicilia, G. et al. Programmable polymer-DNA hydrogels with dual input and multiscale responses. *Biomater. Sci.* **2**, 203–211 (2014).
29. Fern, J. & Schulman, R. Modular DNA strand-displacement controllers for directing material expansion. *Nat. Commun* **9**, 3766 (2018).
30. Cangialosi, A. et al. DNA sequence-directed shape change of photopatterned hydrogels via high-degree swelling. *Science* **357**, 1126–1129 (2017).
31. Hajian, R. et al. Detection of unamplified target genes via CRISPR–Cas9 immobilized on a graphene field-effect transistor. *Nat. Biomed. Eng* **3**, 427–437 (2019).
32. Teng, F. et al. Enhanced mammalian genome editing by new Cas12a orthologs with optimized crRNA scaffolds. *Genome Biol.* **20**, 15 (2019).
33. Kleinstiver, B. P. et al. Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing. *Nat. Biotechnol.* **37**, 276–282 (2019).
34. Hu, W. K., Wang, Z. J., Xiao, Y., Zhang, S. M. & Wang, J. L. Advances in crosslinking strategies of biomedical hydrogels. *Biomater. Sci.* **7**, 843–855 (2019).
35. Gjorevski, N. et al. Designer matrices for intestinal stem cell and organoid culture. *Nature* **539**, 560–564 (2016).
36. Barker, K. et al. Biodegradable DNA-enabled poly(ethylene glycol) hydrogels prepared by copper-free click chemistry. *J. Biomater. Sci. Polym. Ed.* **27**, 22–39 (2016).
37. Phelps, E. A. et al. Maleimide cross-linked bioactive PEG hydrogel exhibits improved reaction kinetics and cross-linking for cell encapsulation and in situ delivery. *Adv. Mater.* **24**, 64–70 (2012).
38. Harrington, L. B. et al. Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. *Science* **362**, 839–842 (2018).
39. Caliari, S. R. & Burdick, J. A. A practical guide to hydrogels for cell culture. *Nat. Methods* **13**, 405–414 (2016).
40. Gobaa, S., Gayet, R. V. & Lutolf, M. P. Artificial niche microarrays for identifying extrinsic cell-fate determinants. *Methods Cell Biol.* **148**, 51–69 (2018).
41. Previtera, M. L. & Langrana, N. A. Preparation of DNA-crosslinked polyacrylamide hydrogels. *J. Vis. Exp.* **90**, e51323 (2014).
42. Turkevich, J., Stevenson, P. C. & Hillier, J. A study of the nucleation and growth processes in the synthesis of colloidal gold. *Discuss. Faraday Soc* **11**, 55–75 (1951).
43. Wei, X. F. et al. Target-responsive DNA hydrogel mediated "stop-flow" microfluidic paper-based analytic device for rapid, portable and visual detection of multiple targets. *Anal. Chem.* **87**, 4275–4282 (2015).
44. Masters, J. R. & Stacey, G. N. Changing medium and passaging cell lines. *Nat. Protoc.* **2**, 2276–2284 (2007).
45. Lee, H. H., Chou, K. S. & Huang, K. C. Inkjet printing of nanosized silver colloids. *Nanotechnology* **16**, 2436–2441 (2005).
46. Milligan, J. F., Groebe, D. R., Witherell, G. W. & Uhlenbeck, O. C. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* **15**, 8783–8798 (1987).
47. Lin, L. et al. Engineering the direct repeat sequence of crRNA for optimization of FnCpf1-mediated genome editing in human cells. *Mol. Ther.* **26**, 2650–2657 (2018).
48. Liu, P. P. et al. Enhanced Cas12a editing in mammalian cells and zebrafish. *Nucleic Acids Res.* **47**, 4169–4180 (2019).

Acknowledgements

This work was supported by Defense Threat Reduction Agency grant HDTRA1-14-1-0006, the Paul G. Allen Frontiers Group and the Wyss Institute for Biologically Inspired Engineering, Harvard University (J.J.C., H.d.P., L.R.S., A.S.M.). L.R.S. was also supported by CONACyT grant 342369/408970, and N.M.A.-M. was supported by MIT-TATA Center fellowship 2748460. We thank C. Johnston for helpful comments during manuscript editing.

Author contributions

R.V.G., H.d.P., M.A.E., L.R.S., P.Q.N., A.S.M. and N.M.A.-M. designed and conducted experiments described in this article and wrote the manuscript. J.J.C. directed the overall research and edited the manuscript.

Competing interests

R.V.G., H.d.P., M.A.E., L.R.S., P.Q.N., A.S.M., N.M.A.-M. and J.J.C. are inventors on U.S. Patent Application No. 16/778,524, which covers CRISPR-responsive materials. J.J.C. is a co-founder and director of Sherlock Biosciences.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41596-020-0367-8.

Correspondence and requests for materials should be addressed to J.J.C.

Peer review information *Nature Protocols* thanks Chase Beisel, Cole DeForest and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 29 December 2019; Accepted: 28 May 2020;
Published online: 17 August 2020

Related links

Key references used in the development of this protocol

English, M. A. et al. *Science* **365**, 780–785 (2019): https://doi.org/10.1126/science.aaw5122
Gootenberg, J. S. et al. *Science* **356**, 438–442 (2017): https://doi.org/10.1126/science.aam9321
Pardee, K. et al. *Cell* **165**, 1255–1266 (2016): https://doi.org/10.1016/j.cell.2016.04.059

nature research

Corresponding author(s): James J Collins

Last updated by author(s): Apr 14, 2020

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☒	☐ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☒	☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☒	☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☒	☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

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	We provide a simple script (running on Arduino 1.8) for data acquisition using the optional RFID modification of the µPAD.
Data analysis	Non applicable

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

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

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data generated or analysed during this study are included in this published article.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We did not perform sample size estimations. All experiments described in this protocol were conducted with at least n=3 technical replicates per sample.
Data exclusions	No data were excluded from the analysis.
Replication	Once established, the procedures presented in this protocol were all performed at least twice with minor variations. All attempts were successful.
Randomization	No randomization was performed.
Blinding	In our original research article [Science 365:780-785 (2019)], the investigator was blinded to group allocation for the detection of RNA molecules following procedure 6 described here.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging