Nanoscale robots exhibiting quorum sensing

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Abstract

Multi-agent systems demonstrate the ability to collectively perform complex tasks—e.g., construction[1-2], search[3], and locomotion[4,5]—with greater speed, efficiency, or effectiveness than could a single agent alone. Direct and indirect coordination methods allow agents to collaborate to share information and adapt their activity to fit dynamic situations. A well-studied example is quorum sensing (QS), a mechanism allowing bacterial communities to coordinate and optimize various phenotypes in response to population density. Here we implement, for the first time, bio-inspired QS in robots fabricated from DNA origami, which communicate by transmitting and receiving diffusing signals. The mechanism we describe includes features such as programmable response thresholds and quorum quenching, and is capable of being triggered by proximity of a specific target cell. Nanoscale robots with swarm intelligence could carry

out tasks that have been so far unachievable in diverse fields such as industry, manufacturing and medicine.

Quorum Sensing (QS) is a well-studied example of collective behavior[6]. This mechanism of cell-cell communication in bacteria utilizes secreted signal molecules to coordinate the behavior of the group. Linking signal concentration to local population density enables each single bacterium to measure population size. This ability to communicate both within and between species is critical for bacterial survival and interaction in natural habitats and has likely appeared early in evolution. Detection of a minimal threshold of signal molecules, termed autoinducers, triggers gene expression and subsequent behavior response. Using these signaling systems, bacteria synchronize particular behaviors on a population-wide scale and thus function as multicellular organisms[6-9].

QS-inspired approaches have been adopted in artificial systems, including mobile robots[10] and wireless sensor networks[11], and naturally occurring genes have been harnessed in synthetic biology to implement QS at the cellular level[12].

Recently we reported a new type of nanoscale robot, fabricated from DNA origami[13], which logically actuates between “off” and “on” states[14-15]. By using various types of DNA logic based on aptamer recognition, toehold-mediated strand displacement[15], etc., these robots can be programmed to respond to diverse stimuli and either present or sequester molecular payloads anchored to the inside of the device. In the present study we aimed to program the robots to exhibit collective behavior, taking advantage of the more elaborate modes of control that such behaviors enable.

The basis for collective behaviors is communication between agents, and QS was chosen as a simple, programmable mechanism to establish it. We designed and constructed a bio-inspired QS system based on an autoinducer whish is released by each individual robot into the environment **(Figure 1a)**. The concentration of this signal is thus proportional to the robot population size, and each individual robot is able to detect it and respond in a concentration-dependent fashion. To achieve this, a molecule previously utilized as a "key" to open the robot, recombinant human platelet-derived growth factor (PDGF)[14-15], was used as an autoinducer. PDGF was loaded into the robot using a peptide tether cleavable by matrix metalloproteinase (MMP)-2 **(Fig. 1a)**. Thus, MMP-2 was set as an external signal initiating QS, and changing its activity enabled us to tune the rate of autoinducer release. Importantly, due to the hollow cylindrical shape of the robot, MMP-2 can freely diffuse in and out of the robot and operate inside it in both its closed and open states, while PDGF has to be cleaved and released from the robot by MMP-2, in order for other robots to sense and respond to it.

The autoinducer release mechanism can be potentially adapted to any environment. For example, one could exploit the inherent instability of RNA for the gradual release of signal from the robots. Alternatively, a UV-cleavable tether would release the signal only upon exposure of the robots to sunlight or another direct source of UV radiation. Choosing enzymes such as MMPs as releasing factors has a therapeutic rationale, as it only initiates QS where enzyme activity is enriched, such as around or directly on metastasizing tumors[16].

The closed robots are hollow shells enabling small molecules such as proteins to freely diffuse in and out of them. Specifically here, the protein diffusing in and out is the release

factor MMP-2, which when inside releases PDGF (tethered to the robot by the MMP-2 substrate polypeptide). The released PDGF can now also freely diffuse out of the robot, and build up a concentration of PDGF in the environment. In contrast, any attached payload (e.g. reporter molecule or unreleased PDGF) is only accessible to beads or other solid phase-based assays when the robot is open. Therefore, all robots – closed and open – participate in generating the PDGF concentration in the environment, but only the open robots contribute to the detectable signal. Robots loaded with auotoinducers were placed in MMP-2-containing buffer at various population densities (from 29 to 18,000 pM). Population density-dependent activation of the robots was demonstrated using both flow cytometry and dynamic light scattering analysis **(Fig. 1b-c)**. Flow cytometry clearly showed distinct, QS-driven robot activation behavior displayed between the constitutively off and constitutively on curves **(Fig. 1c)**.

Engineered QS enables the tuning of response thresholds to fit various conditions or desired behaviors. Here this was achieved by modifying the aptamer gate that responds to the QS signal. In the robot, the aptamer that binds the autoinducer is normally hybridized to a partially-complementary strand, from which it displaces in the presence of the signal as previously shown[14-15]. By changing the number of mismatches in the complementary strand, displacement can be made to occur at lower signal concentrations and with faster kinetics. We used this approach to successfully alter QS-driven behavior in robots **(Fig. 2a)**.

Our QS system can be tuned also via quorum quenching (QQ), by neutralizing or sequestering the autoinducer. To achieve QQ, we used a neutralizing anti-PDGF antibody[14] that effectively negated PDGF binding to its aptamer on the robot, causing

robots to switch to off even though their concentration was high enough to induce QS-driven activation **(Fig. 2b)**. The efficacy of QQ depended on the ability of the neutralizing antibody to compete with the aptamers for autoinducer binding.

We next loaded the robots with antibody Fab' fragments for the human receptor Siglec-7 (CDw328), whose cross-linking on leukemic cells induces growth arrest leading to apoptosis[17]. Jurkat cells (leukemic T cells) were chosen as target cells as they express Siglec-7[18] and also exhibit high levels of MMP-2 activity after activation with cytokines[19]. The cells were treated with varying concentrations of QS-regulated robots for 24 hours. Cell cycle analysis demonstrated cell-triggered QS leading to robot activation and subsequent growth arrest, as no other releasing factor was added to the medium **(Fig 3)**. This highlights the potential of QS as an artificial therapeutic control mechanism that could be utilized in a variety of conditions, given that the proper system is designed with a target-associated releasing factor in mind, such as tumor-derived proteases, bacterial restriction nucleases, etc. A library of autoinducer tethers, each cleavable by a different signal, could be constructed to fit specific needs and environmental conditions.

In this work we implement, for the first time, collective behavior in molecular robots using a bio-inspired mechanism. The design presented here bears many similarities to bacterial QS, while carrying additional features such as the ability to be activated in response to chosen stimuli. Our work also provides a platform for the engineering of more elaborate communication schemes utilizing several sub-populations differing in autoinducer type and response thresholds, with desirable features as control systems for therapeutics and manufacturing.

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Conflict of interest

The authors declare competing financial interest: Y. A., A. A-H. and I. B. are employees of Augmanity Nano Ltd, a for-profit research organization studying applications of DNA nanotechnology..

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Figure 1

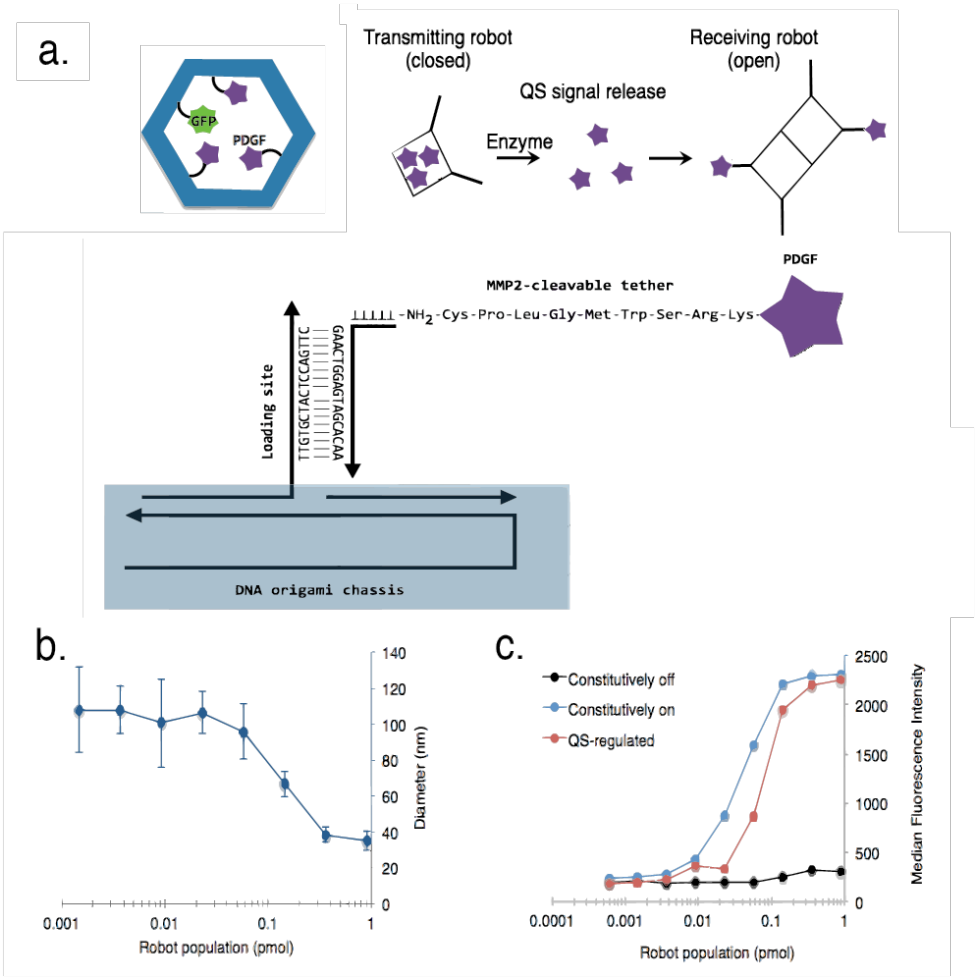
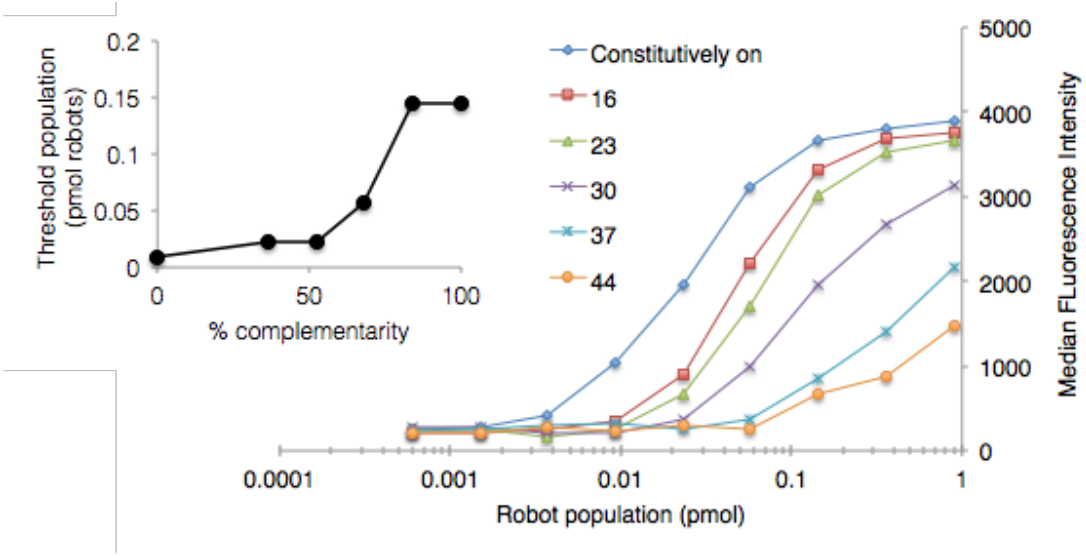


Figure 1: QS in DNA robots. 1A, Schematic design of QS system. PDGF was linked chemically to an MMP2-cleavable peptide tether, to form the autoinducer. This conjugate was further linked to a DNA sequence complementary to the DNA origami-associated loading site sequence (bottom). A mixture of autoinducer and GFP was loaded inside the robot (top left, seen from the side). MMP2 releases the autoinducers from a transmitting robot (in a closed state), these reach a receiving robot, switching it from closed to open (top right). 1B-C, Population-dependent behavior of QS robots. Robots were placed in MMP2-containing buffer in various population sizes in a fixed volume and their state was monitored using dynamic light scattering (1B) or flow cytometry (1C), using beads coated with anti-GFP antibodies.

Figure 2

a)



b)

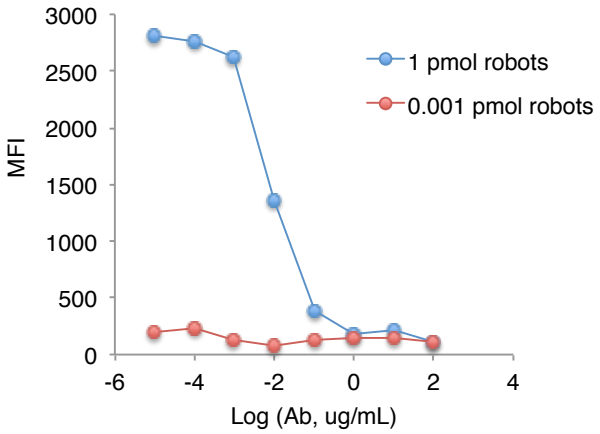


Figure 2: Tuning the behavior of QS robots. QS behavior can be tuned through either the autoinducer-sensing mechanism or through sequestering the autoinducer itself. 2A, reducing complementarity between the DNA strands comprising the robot gate enables to tune the threshold and kinetics of QS behavior. Each curve corresponds to a number of matching bases (max. complementarity: 44 bases; min. complementarity: 16 bases). Inset shows quantitative link between % complementarity and the threshold population of

robots, i.e. the first population with detectable effect. 2B, sequestration of the autoinducer by a neutralizing anti-PDGF antibody enables quorum quenching (QQ).

Figure 3

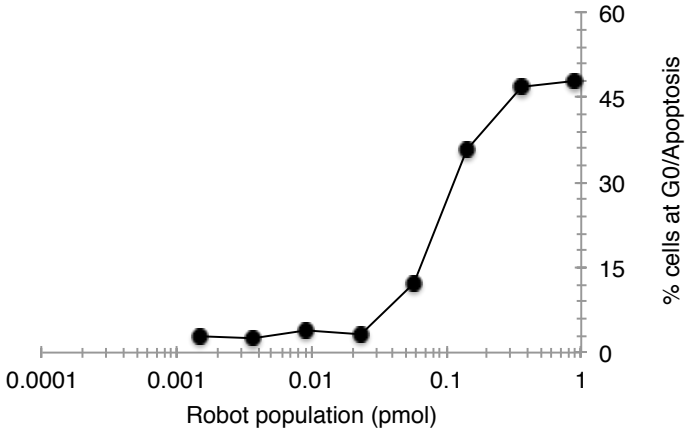


Figure 3: Target cell-triggered QS. Cytokine-activated Jurkat T cells were treated with QS robots loaded with a growth-suppressing antibody (anti-Siglec-7). No MMP-2 was added to the medium as in the previous experiments. QS was driven by MMP2 released from the target cells, leading to subsequent growth arrest. Cells were fixed after treatment and analyzed for cell cycle distribution by flow cytometry.

Supplementary Notes

Supplementary Note 1: robot design and fabrication

DNA origami robots were designed using caDNAno (http://www.cadnano.org) and fabricated as previously described. The M13mp18 circular ssDNA was used as scaffold strand. Staple strands were ordered from Integrated DNA technologies.

Supplementary Table 1: M13mp18 sequence

TAGTTGCATATTTAAAACATGTTGAGCTACAGCATTATATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAG
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Supplementary Table 2: Staple sequences

ID	Description	Sequence
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4	Core	CTTTTGCTTAAGCAATAAAGCGAGTAGA
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6	Core	GGAAGAGCCAAACAGCTTGCAGGGAACCTAA
7	Core	AAAATCACCGGAAGCAAACTCTGTAGCT
8	Core	CCTACATGAAGAACTAAAGGGCAGGGCGGAGCCCCGGGC
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32	Core	ACATTCAGATAGCGTCCAATATTCAGAA
33	Core	AAACATCTTTACCCTCACCAGTAAAGTGCCCGCCC
34	Core	GAGATGACCCTAATGCCAGGCTATTTTT
35	Core	TCCTGAATTTTTTGTTTAACGATCAGAGCGGA
36	Core	GCCGAAAAATCTAAAGCCAATCAAGGAAATA
37	Core	AGCGTAGCGCGTTTTCACAAAATCTATGTTAGCAAACGAACGCAACAAA
38	Core	ACCAATCGATTAAATTGCGCCATTATTA
39	Core	ATCTTACTTATTTTCAGCGCCGACAGGATTCA
40	Core	CCCTAAAAGAACCCAGTCACA
41	Core	GGAAGGGCGAAAATCGGGTTTTTCGCGTTGCTCGT
42	Core	CAGACCGGAAGCCGCCATTTTGATGGGGTCAGTAC
43	Core	TAATATTGGAGCAAACAAGAGATCAATATGATATTGCCTTTA
44	Core	TTCCTTATAGCAAGCAAATCAAATTTTA
45	Core	ACTACGAGGAGATTTTTTCACGTTGAAACTTGCTTT
46	Core	AAACAGGCATGTCAATCATATAGATTCAAAAGGGTTATATTT
47	Core	AACAGGCACCAGTTAAAGGCCGCTTTGTGAATTTCTTA
48	Core	TTCCTGAGTTATCTAAAATATTCAGTTGTTCAAATAGCAG
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50	Core	TTGAGGGTTCTGGTCAGGCTGTATAAGC
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53	Core	AATAAGTCGAAGCCCAATAATTATTTATTCTT
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55	Core	TTTCATAGTTGTACCGTAACACTGGGGTTTT
56	Core	AGGAGCGAGCACTAACAACTAAAACCCTATCACCTAACAGTG
57	Core	CAAAGTATTAATTAGCGAGTTTCGCCACAGAACGA
58	Core	TGGGGAGCTATTTGACGACTAAATACCATCAGTTT
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61	Core	TAATGGTTTGAAATACGCCAA
62	Core	CGGAACAAGAGCCGTCAATAGGCACAGACAATATCCTCAATC
63	Core	ATTAAAGGTGAATTATCAAAGGGCACCACGG
64	Core	GGCAACCCATAGCGTAAGCAGCGACCATTAA
65	Core	AGAAACGTAAGCAGCCACAAGGAAACGATCTT
66	Core	AGAGGTCTTTAGGGGGTCAAAAGGCAGT
67	Core	GGGGACTTTTTCATGAGGACCTGCGAGAATAGAAAGGAGGAT
68	Core	TTTTAGAACATCCAATAAATCCAATAAC
69	Core	AAATGTGGTAGATGGCCCGCTTGGGCGC
70	Core	ACGGATCGTCACCCTCACGATCTAGAATTTT
71	Core	CGCCATAAGACGACGACAATAGCTGTCT
72	Core	GCGTATTAGTCTTTAATCGTAAGAATTTACA
73	Core	AGAGAACGTGAATCAAATGCGTATTTCCAGTCCCC
74	Core	AACGAAAAAGCGCGAAAAAAAGGCTCCAAAAGG

75	Core	TAATTTAGAACGCGAGGCGTTAAGCCTT
76	Core	ACCAGGCGTGCATCATTAATTTTTTCAC
77	Core	CAGCCTGACGACAGATGTCGCCTGAAAT
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79	Core	CTCGAATGCTCACTGGCGCAT
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89	Core	TGGCGAAGTTGGGACTTTCCG
90	Core	CAGTGAGTGATGGTGGTTCCGAAAACCGTCTATCACGATTTA
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92	Core	CTGTATGACAACTAGTGTCGA
93	Core	ATCATAAATAGCGAGAGGCTTAGCAAAGCGGATTGTTCAAAT
94	Core	TTGAGTAATTTGAGGATTTAGCTGAAAGGCGCGAAAGATAAA
95	Core	ATAAGAATAAACACCGCTCAA
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98	Core	AAATAGGTCACGTTGGTAGCGAGTCGCGTCTAATTCGC
99	Core	CAGTATAGCCTGTTTATCAACCCCATCC
100	Core	TTGCACCTGAAAATAGCAGCCAGAGGGTCATCGATTTTCGGT
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108	Core	ACATCGGAAATTATTTGCACGTAAAAGT
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110	Core	AAATCGTACAGTACATAAATCAGATGAA
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114	Core	AGCTGCTCGTTAATAAAACGAGAATACC
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121	Core	GGCTTGCGACGTTGGGAAGAACAGATAC
122	Core	TAAATGCCTACTAATAGTAGTTTTCATT
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124	Core	TGACCATAGCAAAAGGGAGAACAAC
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127	Core	AGACGCTACATCAAGAAAACACTTTGAA
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129	Core	GATTCCTGTTACGGGCAGTGAGCTTTTCCTGTGTGCTG
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218	Edge	TTTTTTTTTTTTTTTTCCTCGTTAGAATCAGAGCGTAATATC
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221	Edge	TTTTTTTTTTTTTTTAACCGCCTCCCTCAGACCAGAGC

222	Edge	TCTGACAGAGGCATTTTCGAGCCAGTTTTTTTTTTTTTTT
223	Edge	TTTTTTTTTTTTTTTTTTCAGCGGAGTTCCATGTCATAAGG
224	Edge	TTTTTTTTTTTTTTTCGCCCACGCATAACCG
225	Edge	AATTACTTAGGACTAAATAGCAACGGCTACAGATTTTTTTTTTTTTTT
226	Edge	CAAGTTTTTTGGTTTTTTTTTTTTTTT
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228	Edge	TTTTTTTTTTTTTTTTGAATCGGCCGAGTGTTGTTTTTTTTTTTTTTTT
229	Edge	TTTTTTTTTTTTTTCATCTTTGACCC
230	Edge	TTTTTTTTTTTTTATAATCAGAAAATCGGTGCGGGCCTTTTTTTTTTTTT
231	Edge	GATACAGGAGTGTACTTTTTTTTTTTTTTTT
232	Edge	TTTTTTTTTTTTTTTGGCGCAGACAATTTCAACTTTTTTTTTTTTTTT
233	Edge	GGAGGTTTAGTACCGCTTTTTTTTTTTTTTT
234	Edge	TTTTTTTTTTTTTACCGCCAGCCATAACAGTTGAAAGTTTTTTTTTTTTT
235	Edge	TTTTTTTTTTTTTTTATAGCAATAGCT
236	Handles	AATAAGTTTTGCAAGCCCAATAGGGGATAAGTTGTGCTACTCCAGTTC
237	Handles	ACATAGCTTACATTTAACAATAATAACGTTGTGCTACTCCAGTTC
238	Handles	CCTTTTTGAATGGCGTCAGTATTGTGCTACTCCAGTTC
239	Handles	CGTAACCAATTCATCAACATTTTGTGCTACTCCAGTTC
240	Handles	CACCAACCGATATTCATTACCATTATTGTGCTACTCCAGTTC
241	Handles	CCACCCTCATTTTCTTGATATTTGTGCTACTCCAGTTC
242	Handles	AACTTTGAAAGAGGAGAAACATTGTGCTACTCCAGTTC
243	Handles	CAAGGCGCGCCATTGCCGGAATTGTGCTACTCCAGTTC
244	Handles	CATAGCCCCCTTAAGTCACCATTGTGCTACTCCAGTTC
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246	Handles	AACGGTGTACAGACTGAATAATTGTGCTACTCCAGTTC
247	Handles	GATTCGCGGGTTAGAACCTACCATTTTGTTGTGCTACTCCAGTTC
248	Guides	AGAGTAGGATTTCGCCAACATGTTTTAAAAACC
249	Guides	ACGGTGACCTGTTTAGCTGAATATAATGCCAAC
250	Guides	CGTAGCAATTTAGTTCTAAAGTACGGTGTTTTA
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252	Guides	AAGCCAACGGAATCTAGGTTGGGTTATATAGATTAAGCAACTG
253	Guides	TTTAACAACCGACCCAATCGCAAGACAAAATTAATCTCACTGC
254	Guides	TTTAGGCCTAAATTGAGAAAACTTTTTCCTTCTGTTCCTAGAT
255	Guides Removal	GGTTTTTAAAACATGTTGGCGAAATCCTACTCT
256	Guides Removal	GTTGGCATTATATTCAGCTAAACAGGTCACCGT
257	Guides Removal	TAAAACACCGTACTTTAGAACTAAATTGCTACG
258	Guides Removal	AACGCTCATTTCAAGATCAGCATTTACATTTAACGCATTAAGC
259	Guides Removal	CAGTTGCTTAATCTATATAACCCAACCTAGATTCCGTTGGCTT
260	Guides Removal	GCAGTGAGATTAATTTTGTCTTGCGATTGGGTCGGTTGTTAAA
261	Guides Removal	ATCTAGGAACAGAAGGAAAAAGTTTTCTCAATTTAGGCCTAAA

To fold the robots, scaffold and staple DNA were mixed at a ratio of 1:10, respectively, in Tris-Acetate-EDTA buffer supplemented with 10 mM $MgCl_2$. The mixture was subjected to a temperature-annealing ramp in the following sequence: 1) from 85°C to 60° C, 5 min/°C; 2) from 60 °C to 25 °C, 75 min/°C. Subsequently, excess staples were

removed by centrifugal filtration using Amicon Ultra-0.5mL 100K MWCO centrifugal filters (Millipore).

Payload synthesis

GFP was fused to loading-sequence DNA (5AmMC6/GAACTGGAGTAGCAC Integrated DNA Technologies) by EDC conjugation according to the manufacturer's instructions. Anti-human p75/AIRM Fab' fragments were obtained by digesting whole IgG using a Fab' generation kit (Pierce) according to the manufacturer's instructions. After purification, Fab' fragments were fused to loading sequence DNA by EDC conjugation.

Robot loading and purification

100 pmol folded robots were loaded with autoinducer and payload (at a 3:1 ratio) by incubation at a 5-fold molar excess of mixture to loading sites. Loading was performed for 2 hours on a rotary shaker at room temperature in folding buffer (10 mM $MgCl_2$ in 1X TAE). Finally, loaded robots were cleaned by centrifugal filtration with a 100K MWCO Amicon column (Millipore) as described above.

Loading in this design was done stochastically. However, by redesigning the loading site sequences and autoinducer/payload specificities, loading can be directed to specific sites. However, stochastic loading was effective (albeit potentially less optimal than directed loading), for the following reasons: a) robots containing only autoinducer can serve as autoinducer sources indicating population size; b) robots containing only payload (GFP/Fab') respond to external autoinducer and contribute to the readout; and c) robots containing both serve both functions.

Supplementary Note 2: QS system design

5'-amine-modified linker oligonucleotide (5AmMC6/TTTTTGAACTGGAGTAGCAC, Integrated DNA Technologies) was conjugated using the heterobifunctional crosslinker SMCC to the C-terminal thiol group in Lys-Pro-Leu-Gly-Met-Trp-Ser-Arg-Cys (custom ordered from American Peptide Company), containing the cleavage site of MMP-2. according to the manufacturer instruction, at a DNA:peptide ratio of 1:2. After quenching with 2-mercaptoethanol and purification, the oligonucleotide-peptide hybrid was further conjugated with PDGF using EDC crosslinking, purified and verified with spectrophotometry to yield to complete autoinducer. The cleaved autoinducer maintained its ability to bind to anti-PDGF antibodies as well as to the PDGF aptamer.

Kinetics of peptide cleavage by MMP-2 was measured by fluorometry using a fluorogenic MMP-2/MMP-2 substrate (5 μM) and human recombinant MMP-2 in assay buffer (Tris-EDTA containing 150 mM NaCl, 10 mM $MgCl_2$ and 1 μM $ZnSO_4$, pH 7.5) at room temperature. The desired concentration of MMP-2 for this study was fixed at 5 μg/mL **(Fig. S1)**.

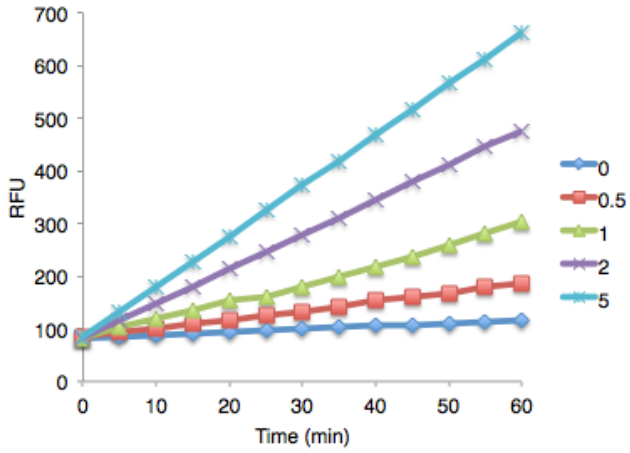

Fig. S1: MMP-2 calibration assay, used to determine desired MMP-2 concentration for the purpose of activating QS in robots for this study (see above for detail).

To evaluate the kinetics of autoinducer release from robots, autoinducer-loaded robots were exposed to MMP-2 (5 μg/mL) for 1 h at room temperature, after which the samples were measured directly in a PDGF ELISA **(Fig. S2).**

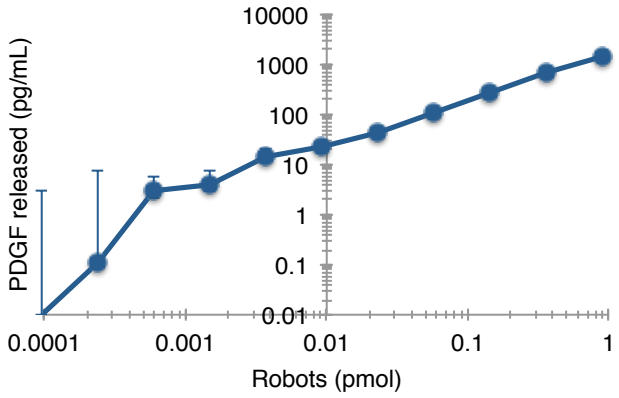

Fig. S2: Autoinducer release from MMP-2 treated robots.

Supplementary Note 3: Cell culture

Jurkat cells were obtained from American Type Culture Collection (ATCC) and maintained at 37 deg. and 5% CO_2 in RPMI 1640 containing 10% fetal calf serum. Prior to incubation with robots, cells were diluted to a density of 100,000 cells/mL in 96 well plates and activated with 200 ng/mL of recombinant human IL-6 (Peprotech) overnight.
Following activation, the cells were treated with varying concentrations of either free anti-p75/AIRM Fab' fragments (cross-linked by 25 ug/mL secondary anti-mouse IgM), or the equivalent amount of Fab' fragments loaded into QS-regulated robots, for 24 hours. Following this period, the cells were analyzed for cell cycle distribution using propidium iodide as previously described.

Supplementary Note 4: Dynamic light scattering and flow cytometry

Dynamic light scattering was performed using a Malvern Zetasizer Nano instrument using various concentrations of robots in Tris-EDTA buffer supplemented with 10 mM $MgCl_2$. The minimal robot concentration that enabled reliable detection (based on good correlation function) was 29 pM, and the results obtained were good as a qualitative confirmation of QS-driven switch from closed to open state.
The advantage of flow cytometry is the use of target-coated microspheres, which isolate from any population only the robots that open and directly bind them, allowing much more reliable measurements and at lower population densities. Flow cytometry was performed using an Accuri C6 flow cytometer equipped with 488 nm and 640 nm lasers, and analyzed with FlowPlus software. Cell cycle analysis was done using propidium iodide as previously described.