Macrophage calcium reporter mice reveal immune cell communication *in vitro* and *in vivo*

Highlights
- A reporter mouse for measuring cellular calcium dynamics in macrophages
- Analysis pipeline infers cell-cell communication from correlated calcium dynamics
- Immune-stimulatory DNA promotes correlated calcium dynamics *in vitro*
- Spontaneous coordination between cells is detected *in vivo*

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In brief
Taghdiri et al. utilize a macrophage reporter mouse to infer immune cell communication based on correlated cellular calcium dynamics *in vitro* and *in vivo*. 

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Macrophage calcium reporter mice reveal immune cell communication in vitro and in vivo

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SUMMARY

Cell communication underlies emergent functions in diverse cell types and tissues. Recent evidence suggests that macrophages are organized in communicating networks, but new tools are needed to quantitatively characterize the resulting cellular conversations. Here, we infer cell communication from spatiotemporal correlations of intracellular calcium dynamics that are non-destructively imaged across cell populations expressing genetically encoded calcium indicators. We describe a hematopoietic calcium reporter mouse (Csf1rCreGCaMP5f) and a computational analysis pipeline for inferring communication between reporter cells based on “excess synchrony.” We observed signals suggestive of cell communication in macrophages treated with immune-stimulatory DNA in vitro and tumor-associated immune cells imaged in a dorsal window chamber model in vivo. Together, the methods described here expand the toolkit for discovery of cell communication events in macrophages and other immune cells.

INTRODUCTION

Cell communication underlies emergent functions such as cognition in the brain (Hahn et al., 2019), metabolism in the liver (Xiong et al., 2019), and contraction in the heart (Tirziu et al., 2010). It productively coordinates tissue morphogenesis during development, but it also maladaptively spreads cell damage after injury (Ablasser et al., 2013; Ellison et al., 2016; Garcia-Dorado et al., 2004; Parthasarathi et al., 2006; Patel et al., 2009).

Detection of information transfer between non-excitable cells requires a high degree of suspicion and experimental serendipity when investigated using destructive, low-throughput, and low-temporal-resolution assays (Browaeys et al., 2020; Rieckmann et al., 2017; Shalek et al., 2014). However, once discovered, mechanisms of communication such as mechanical coupling, transmembrane signaling, gap junction communication, secreted cytokines, or extracellular vesicles can be systematically explored with molecular specificity using genetic and pharmacologic perturbations as well as co-cultures or chimeric mouse models (La-doux and Mege, 2017; Leybaert et al., 2017; Peyret et al., 2019). Since discovery is currently a barrier, we set out to develop tools for recognition and quantification of cell communication events in non-excitable cells.

Macrophages are non-excitable innate immune cells in virtually every tissue (Epelman et al., 2014). Resident and bone marrow derived macrophages patrol local tissue microenvironments,
remove dead cells after injury, mount innate and adaptive anti-
pathogen responses during infections, and facilitate immune recog-
nition of cancer (Honold and Nahrendorf, 2018; Pittet et al., 2018). Morphologic evidence suggests that macrophages are
arranged as networks of cells and that they communicate with parenchymal cells such as in the heart, where they influence cardiac conduc-
tion (Hulsmans et al., 2017). Existing methods for studying communication between macrophages include transfer of microinjected or scrape-loaded membrane-impermeant dyes, fluorescence recovery after photo bleaching, measurement of electrical conductance via patch clamping, or dynamic intracel-
lar imaging of fluorescent reporter molecules (Abbaci et al., 2008).

Calcium represents an attractive indicator of cell communica-
tion in macrophages because it is a dynamic second messenger
influenced by multiple signaling pathways. In non-communicating
populations of cells, calcium dynamics are not necessarily corre-
lated. We reasoned that non-destructive monitoring of calcium
dynamics in a population of cells and detection of their spatiotem-
poral correlations could be used to infer cell communication, even
if the molecular stimuli, mediators, and mechanisms were unknown.
Calcium can be measured non-destructively by imaging exo-
genously loaded dye in cell culture (Grienberger and Konnerth,
2012; Scenes et al., 1998) or in intact tissue slices (Svoboda et al.,
1997), but these reagents are not reproducibly loaded in vivo.
Genetically encoded calcium indicators (GECIs) have emerged
durable non-destructive longitudinal reporters of calcium dy-
namics in populations of cells both in vitro and in vivo. The
methods for quantification and the number of available GECIs is
rapidly expanding (Berens et al., 2018; Chen et al., 2012; Deneux
et al., 2016; Inoue et al., 2019; Mues et al., 2013; Mukamel et al.,
2009; Perez Koldenkova and Nagai, 2013; Pnevmatikakis et al.,
2008). Although absolute quantification of calcium concentra-
tions can be obtained using ratiometric GECIs, single-wavelength
GECIs are sufficient for quantifying relative fluorescence dy-
namics (Svoboda et al., 1997; Ulbricht et al., 2021).

Here, we describe a method for detecting cell communication
in macrophages and other immune cells using a hematopoietic
calcium reporter mouse (Csf1r-CreGCaMP5) and a computational
pipeline that infers communication locations and times by quan-
tifying “excess synchrony.” We show that these methods allow
discovery and quantification of cell communication in DNA-stim-
ulated macrophages in vitro and peri-tumor immune cells in vivo.
Together, the methods expand our toolkit for discovering and
quantitatively investigating immune intercellular communication.

RESULTS

Immune calcium reporter mouse construction and
characterization

To enable non-destructive quantification of calcium dynamics in
macrophages, we bred mice expressing Cre recombinase under the
transcriptional regulation of the hematopoietic colony-stimu-
Jating factor 1 receptor promoter (Csf1r-Cre) with mice expressing
a Cre-deletable LoxP-flanked STOP sequence separating a
constitutive CAG promoter from the downstream genetically en-
coded calcium indicator (GCaMP5) followed by IRES-tdTomato
as a reference reporter (Figures S1A and S1B) (Wei et al., 2019).

After Cre-mediated excision of the STOP sequence, both
GCaMP5 and tdTomato were expressed in a bicistronic fashion.
Csf1r-Cre was chosen because the Csf1 receptor is expressed
broadly by adult macrophages; however, the reporter will be
activated in all Csf1r-expressing cells and their progeny. We first
evaluated the distribution of expression in adult offspring
(Csf1r-CreGCaMP5) using confocal imaging of the heart, spleen,
kidney, and lung (Figure S1C). We also performed flow cytomet-
ric analysis of peripheral blood leukocytes and cells released from
digested solid organs (Figures S1D–S1F). This revealed
tdTomato expression in multiple hematopoietic lineages, allow-
ing calcium dynamics to be screened across a broad range of
hematopoietic subsets and tissue resident macrophages. We
focused on macrophages because we and others have found
them to be morphologically organized as potentially communi-
cating networks in solid organs and because recent studies in
the heart showed that they functionally alter cell communication
during cardiac conduction (Hulsmans et al., 2017).

Calcium dynamics of macrophage cell death induced by
DNA sensing

We first examined the Csf1r-CreGCaMP5 reporter cells in vitro.
Bone marrow-derived macrophages (BMDMs) were iso-
lated, differentiated with macrophage colony-stimulating factor
(m-CSF), and serially imaged using time-lapse fluorescence mi-
croscopy in an environmentally controlled chamber. This
enabled non-destructive imaging at single-cell resolution over
long durations and at multiple locations, but with limited sam-
ping frequency. To define baseline calcium signals we imaged
for 48 h at a sampling interval of 2 min and observed negligible
changes in fluorescence (Figure 1A; Video S1A). We next evalu-
ated freely diffusible innate immune stimuli such as lipopolysac-
charides (LPS) or cyclic di-GMP but again observed no
response. However, when we stimulated cells with double-
stranded DNA (dsDNA) (Herring Testis DNA, ~2 kbp) complexed
with a polyelectrolyte transfection reagent (Lipofectamine 2000;
Invitrogen), hereafter termed dsDNA, the macrophages ex-
hibited marked fluorescence changes, as demonstrated by
maximum image projections (MIPs), percentage fluorescence
area, and number of fluorescent cells (n = 6, p < 0.0001) (Figures
1B–1D; Video S1B). At a single-cell level, the calcium responses
were transient (7 ± 3 min in duration), asynchronous (distributed
throughout the entire 48-h observation period) (Figures 1E–1G),
and lacked spatial organization (Figure 1H), all of which are
consistent with the particulate nature of complexed dsDNA
and its need for uptake by cells before engaging by cytosolic
sensors (Patel et al. (2009) #1495).

Inspection of gray-scale differential interference contrast (DIC)
images suggested that each fluorescence increase was followed
closely by changes in cell morphology suggestive of cell death
(Figure 1I). This is consistent with the established ability of
DNA to induce pyroptosis when sensed by the cytosolic innate
immune sensor, absent in melanoma 2 (AIM2) (Horning et al.,
2017). To enable quantitation, we subtracted successive gray-
scale DIC images to create a differential DIC signal that
increased rapidly and then dropped to levels below baseline at
the time of morphologic cell death. By comparing the timing of
reporter fluorescence and differential DIC signals for each
macrophage, we find that calcium overload is followed closely by cell death (Figures 1J and 1K).

**Macrophage cell communication is induced by fatal DNA sensing**

The effects of cell death on neighboring cells are difficult to visualize in live cells and are incompletely understood. In contrast to soluble stimuli, which uniformly affect all cells, complexed dsDNA is particulate in nature and causes asynchronous cell death in directly affected macrophages, which allows surviving neighbor cells to be interrogated for evidence of cell communication. To study neighboring cell responses to DNA-induced cell death, we used scanning confocal microscopy and performed higher-frequency longitudinal calcium reporter imaging at 2 Hz, which we confirmed did not undersample the observed calcium responses (Figure S2; Video S2). As above, macrophages exhibited rare spontaneous calcium elevations at steady state (Video S3A) but exhibited prominent calcium overload in response to DNA stimulation (Video S3B). We noticed that bystander macrophages neighboring the calcium-overloaded cell exhibited fluorescence fluctuations (Figures S3A–S3B). Therefore, we tested whether bystander signals were the result of cell communication that could be inferred from correlated single-cell calcium dynamics.

We developed a computational pipeline that converts raw time-lapse fluorescence images into single-cell impulse trains,
where each impulse represents the timing of a calcium elevation ($T_{Ca}$). Briefly, single-cell regions of interest (ROIs) were defined and background-corrected to yield a collection of single-cell fluorescence time series that could be low pass filtered and subjected to a peak-finding algorithm. Peaks were transformed into binary impulses at each $T_{Ca}$ (Figures 2A and 2B). Cells were sorted based on the timing of their maximum amplitude calcium elevation $T_{CaMax}$ and plotted as a heatmap of single-cell calcium dynamics. This revealed a temporal progression of calcium elevations suggestive of intercellular signal propagation (Figure 2C).

Using the initial calcium-overloaded cell as a reference ($T_0$), we plotted $T_{CaMax}$ for each cell versus its distance from the initiating cell and revealed a propagation velocity of 9 $\mu$m/s, which is consistent with previously reported propagation velocity for intercellular calcium relays (Dieterle et al., 2020; Verma et al., 2018). Inspection of the time-lapse images confirmed that the isolated calcium-overloaded cell precipitates a wave of transient calcium elevations in neighboring cells (Figure 2E). Cross-correlations of temporally ordered calcium elevations from the entire time-lapse recording also predicted communication with similar
timing (Figure 2F). Taken together, these data suggest that DNA-induced macrophage cell death is rapidly communicated to neighboring cells, where it precipitates non-fatal calcium dynamics.

Inference of cell communication from Csf1rCre;GCaMP5f reporter dynamics in vivo

Next, we asked whether the calcium reporter could infer cell communication in the complex and dynamic tissue microenvironments in vivo. To facilitate in vivo imaging, we installed a dorsal window chamber in the Csf1rCre;GCaMP5f reporter mouse and performed time-lapse imaging (Figure 3A; Video S4) (Pittet et al., 2018). We implanted 1 million MC38-H2B-mCherry colon adenocarcinoma cells into the tissue underlying the window chamber to facilitate host immune cell recruitment. Within 24–72 h of MC38-H2B-mCherry cell implantation, we discovered host Csf1rCre;GCaMP5f reporter cells with highly dynamic calcium elevations. Comparisons allow estimation of whether synchrony occurs by chance or exhibits excess synchrony, beyond chance, which we interpret as putative cell communication.

The heatmap (Figure 3B) shows a cluster of cells with temporally localized calcium elevations. Cross-correlation heatmap (Figure 3C) reveals highly localized in time and space. We defined ROIs and quantified each single-cell fluorescence across time. Heatmaps were then created by hierarchically clustering the single-cell dynamics. This revealed a method for quantifying normalized number of calcium elevations (S/w), also known as synchrony, for real and generated single-cell calcium elevation trains, where S is a function of temporal window size, w, and window initiation time, t. (F) Method for defining the timing of excess synchrony of real cell populations compared with the corresponding generated populations.

(G) Heatmap of excess synchrony (ΔS/w) as a function of temporal window size, w, and window initiation time, t. Inset shows timing of high excess synchrony and putative cell communication.
cells with synchronous calcium elevations amid a background of seemingly random calcium elevations, suggesting possible cell communication (Figure 3B). Cross-correlations of temporally ordered calcium elevations revealed similar timing and duration of synchronous calcium elevations (Figure 3C). We therefore set out to determine if the observed synchrony was best explained by cell communication.

**Excess synchrony: A metric for identifying cell communication events in vivo**

To determine whether correlated calcium elevations are due to cell communication or if they can be explained simply by chance, we defined a metric called excess synchrony. The metric quantifies the extent to which calcium elevations in a specific window of time exceed randomly generated calcium elevations sampled from a statistically comparable population of cells. We first used the experimental data to assemble the overall distribution of calcium elevation frequencies (calcium elevations per 5-min recording) and modeled it as a negative binomial (goodness of fit of 2.3 × 10⁻⁸). Next, we sampled from the distribution to create a “generated” population of synthetic cells (Figure 3D). Synchrony was then defined as the number of calcium elevations (S) within a defined region of time (τ). Excess synchrony was defined as (S/ω = (Sexp − Sth)/w), where Sexp was the difference between the amount of experimentally observed synchrony (Sexp) and a threshold of generated synchrony (Sth) chosen to emphasize specificity over sensitivity, and where w was the window of time within which calcium elevations are deemed synchronous (Figure 3E). At the extremes of large and small window sizes, random calcium elevations dominate and S approaches zero; however, at an optimal window size (ωopt), a putative cell communication process creates a local maximum of excess synchrony (S/ω) due to temporally concentrated calcium elevations that exceed the synchrony predicted by generated cells (Figure 3F). To identify cell communication events, we plotted a heatmap of excess synchrony as a function of window size and time. We thresholded it at the 80th percentile to balance specificity over sensitivity, and where the timing of communication (τcomm) to be the midpoint of time points above threshold (Figure 3G).

To validate the predictions, we examined the spatial distribution of synchronous cells at time τ and observed two qualitative groups, one that was highly localized in space and one that was dispersed (Figure 4A). We separated these populations using unsupervised k-means clustering of Euclidean distances (Figure 4B). Heatmaps of normalized fluorescence dynamics showed that localized cells from cluster 1 only had calcium elevations one or two times during the recording, whereas spatially dispersed cells from cluster 2 exhibited calcium elevations more frequently and were likely the cells that appeared synchronous by chance, as predicted above (Figures 4C–4D). Inspection of the time-lapse images confirmed these predictions, as it showed organized waves of calcium fluorescence propagation in cluster 1 but no qualitative evidence of communication in cluster 2 (Figures 4E–4G). Cluster 1 also exhibited different calcium elevation character with significantly higher calcium fluorescence full-width half-maximum (FWHM) and relative calcium elevation amplitude (∆F/F) (Figures 4H–4K). Together, these data demonstrate that excess synchrony can be used to identify cell communication events in vivo amid a background of random calcium elevations.

**Prediction of spatiotemporal cell communication and regulation in a tumor context in vivo**

To enable quantification of reporter fluorescence and inference of putative cell communication in dense communities of reporter cells, we expanded the temporal synchrony pipeline to add spatial resolution. A maximum image intensity plot of an example recording is shown (Figure 5A; Video S5). In this dataset, the dynamics of 522 single cells were quantified using the pipeline above and displayed as a heatmap (Figure 5B). We spatially subset the field into 50 μm × 50 μm sub-images with 25-μm overlap to identify local areas of excess synchrony (Figures S4A–S4B). This resulted in a 3D volume of excess synchrony versus space and time (Figure 5C), which enabled identification, localization, and counting of cell communication events at different levels of stringency. Slices from the volume at locations of excess synchrony are shown as a function of space (Figures 5D and S5), as a function of time (Figures 5F and S5), and as a kymograph in space-time (Figures 5H and S5). We repeated the analysis for several temporal window sizes and synchrony stringencies to show how predicted cell communication events would vary (Figure 5J). The resulting self-similar curves revealed peaks of synchrony when the window size w approximated the characteristic timescale of cell communication. Increasing the stringency by increase the synchrony threshold led to fewer but more prominent cell communication events, as expected. Finally, we compactly displayed the predicted communication events by color-encoding synchrony events on a spatial map which facilitated comparisons to the underlying fluorescence time-series data (Figure 5K).

**DISCUSSION**

We have constructed and characterized a Csf1rCrestGCaMP5β calcium reporter mouse and used it to infer immune cell communication in vitro and in vivo. In vitro, the reporter and the associated analysis pipeline enabled direct visualization of fatal calcium overload precipitated by DNA sensing and rapid non-fatal communication to surviving macrophage neighbors. In vivo, in the context of an MC38-H2B-mCherry tumor (Videos S6A and S6B), it enabled immune cell communication to be inferred from spatiotemporal analysis of Csf1rCrestGCaMP5β calcium elevations. Because cell communication occurs in a background of ambient calcium fluctuations of unknown etiology, we defined a metric termed excess synchrony and an associated computational pipeline to identify putative cell communication events amid random calcium elevations. The tools are highly generalizable and should be applicable to other genetically encoded calcium reporter experiments. Importantly, the inference method involves correlating single-cell calcium dynamics but does not require a priori knowledge of the molecular stimulus, the mediators, or the mechanisms of communication. It is therefore a convenient tool for discovery of communication events. However, once discovered, the inference method can be used to...
probe mechanism by quantifying how cell communication changes in response to genetic or pharmacologic modulation of candidate mediators. For example, one can use co-cultures, chimeric mice, adoptive transfers, bone marrow transplantation, window chamber cell transplantation, or parabiosis to delineate which cell types and mediators are involved in sending versus receiving signals.

The potential mechanisms underlying the observed cell communication are diverse and span length and time scales as well as target site specificity. For example, gap junction intercellular communication (GJIC) enables rapid contact-dependent exchange of intracellular molecules less than 1 kDa between neighboring cells expressing compatible connexins (Leybaert et al., 2017). GJIC can be controlled dynamically through modulation of cell contact and channel gating and thus offers a mechanism for creating functional networks with selected cells in a shared microenvironment. Mechanotransduction also provides a mechanism for rapid relay of signals such as stretch and loss of neighbors in scratch assays (Ladoux and Mege, 2017). At the other extreme, secreted factors such as chemokines, cytokines, and growth factors can be released to travel through the extracellular space through diffusion or convection and target distant cells that express the appropriate cognate receptor (Sonnenberg and Hepworth, 2019).

In our in vitro experiments, the Csf1rCreGCaMP5β reporter enabled real-time visualization of complexed DNA inducing calcium overload and cell death followed by rapid communication to neighboring cells. Although we and others have shown that cytosolic DNA sensing precipitates spread of gap junction-permeable cyclic di-nucleotide second messengers after
cytosolic DNA sensing (Ablasser et al., 2013; Patel et al., 2009), we were unable to induce calcium fluctuations with cell-permeable cyclic-di-GMP in our reporter. This suggests communication is mediated by a different highly diffusive mediator, such as ions, metabolites, or other secondary messengers, communicating via the extracellular space, gap junctions, or an intracellular relay system.

In our in vivo experiments, the possible mechanisms and mediators are diverse, but can be probed using pharmacologic modulators such as selective ion channels blockers or gap junction inhibitors (e.g., carbenoxolone). In addition, to gain more cell type specificity, it would be valuable to cross the GCaMP reporter with more selective Cre-inducing promoter such as S100a8 for neutrophils or Cx3cr1 for monocytes and macrophages.

Finally, at first glance, it may appear to be a weakness that calcium is not unique to a single specific signaling pathway. However, calcium was chosen precisely because it integrates...
inputs from so many different sources and because it has high bandwidth to reflect dynamic cell states faithfully. Here, we use the calcium reporter to discover where and when immune cells are “talking” but we sacrifice knowledge of the specific stimuli and mediators. This is analogous to watching a film in a foreign language, where one can infer which characters are conversing, when, and where, but the detailed meaning of the conversation is unknown. Nevertheless, this is useful because, once communication events are recognized, they can mechanistically probed, as detailed above.

In conclusion, discovery of cell communication in non-excitable cells has historically required serendipity or a high degree of suspicion. Here, we introduce a method for discovery through inference of cell communication via correlated intracellular calcium dynamics of genetically encoded calcium reporter cells. We hope that this addition to the immunologist’s toolkit will shed light on as-yet undiscovered cellular conversations.

Limitations
Our studies use a Csflr-Cre mouse to induce the GCaMP5 reporter. Doing so activates the reporter in a broad range of hematopoietic cells. In vitro, this is not limiting because we isolate and differentiate bone marrow-derived cells into macrophages; however, in vivo, the communicating cell types are less well defined. To gain more cell type specificity in future experiments, it would be useful to cross calcium reporters with more selective Cre-inducing promoters such as S100a8-Cre for neutrophils or Cx3cr1-Cre for monocytes and macrophages. A second limitation is that we cannot immediately validate our predicted cell communication events because our method is a discovery tool. Only if the correlated dynamics are altered by perturbing specific cell communication mechanisms can we validate the predictions. While some of the observed communication events in vitro and in vivo are self-evident by inspection of correlated signaling, others are less certain because they are inferred from within highly dynamic and densely packed in tissue. Application of mutual information theory and causal inference may be helpful for determining whether correlated calcium dynamics are cell autonomous or the result of cell-to-cell versus environment-to-cell communication (Quinn et al., 2011; Schreiber, 2000; Wibral et al., 2013). The need to identify where and when cell communication occurred was a source of inconvenience during our study due to the limited field of view; however, addition of widefield time-lapse imaging will likely improve communication event detection efficiency in future studies. Finally, our current studies are limited by the bandwidth of the GCaMP5 calcium reporter. Fortunately, new variants of calcium reporters continue to be developed and such advances will be immediately compatible with the proposed experimental and computational pipeline.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- Materials availability
- Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- Animals
- METHOD DETAILS
- Tissue processing
- Flow cytometry
- Cell culture
- Time-lapse imaging of calcium reporter dynamics in vitro and in vivo
- Quantitative analysis of epifluorescence imaging
- Inference of cell communication from calcium reporter time lapse imaging
- Spatiotemporal excess synchrony pipeline details
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2021.100132.

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AUTHOR CONTRIBUTIONS
N.T. and K.R.K. designed the study, performed all experiments and analysis, and wrote the initial manuscript. D.C. performed flow cytometric analysis. Z.F. and R.K. performed surgeries and intravital imaging. T.P.C. developed analysis methods. All authors (N.T., D.M.C., Z.F., K.H., R.K., R.W., T.P.C., and K.R.K.) analyzed data, synthesized results, and edited the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCES | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| **Antibodies**       |        |            |
| Ly6G - clone 1A8     | BioLegend | Cat #127616, RRID: AB_1877271 |
| CD11b - clone M1/70   | BioLegend | Cat #101226, RRID: AB_830642 |
| NK1.1 - clone PK 136  | BioLegend | Cat #108708 |
| Thy1 - clone 53-2.1   | BioLegend | Cat #140307, RRID: AB_10643585 |
| Ly6C - clone HK1.4    | BioLegend | Cat #128015, RRID AB_1732087 |
| 4',6-diamidino-2-phenylindole (DAPI) | Invitrogen | Cat #D1306 |
| Ter119 - CD90.2       | BioLegend | Cat #116208, RRID: AB_313708 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Phosphate-buffered saline 10X (PBS) | Gibco | 70011044 |
| Bovine serum albumin (BSA) | MP bio | 160069 |
| Red Blood Cell (RBC) Lysing Buffer 10X | Invitrogen | 50-112-9743 |
| DMEM - Dulbecco’s Modified Eagle Medium (1X) | Gibco | 11965-092 |
| DMEM - Dulbecco’s Modified Eagle Medium (1X) | Gibco | 31053-028 |
| Opti-MEM | Gibco | 31985-070 |
| FBS-Fetal Bovine Serum | Corning | 35010CV |
| Penicillin-Streptomycin Solution, 100x | Corning | 30-002-CI |
| Macrophage Colony-Stimulating Factor (Mcsf) | Peprotech | 315-02 |
| Collagen type 1 | Corning | 354236 |
| Lipofectamine 2000 agent | Invitrogen | 11668-030 |
| Herring Testis (HT) DNA | Sigma | D6898 |
| **Experimental models: Organisms/strains** |        |            |
| Mouse: C57CL/6-Tg (Csf1r-cre)1Mnz/J | Jackson Laboratory | 029306 |
| Mouse: B6;129S6-Poi2R2Atn (pb-CAG-GCaMP5g, -tdTomato) Tvd | Jackson Laboratory | 024477 |
| **Software and algorithms** |        |            |
| FIJI                  | National Institute of Health, USA | RRID: SCR_002285 |
| Matlab                | Mathworks | R2020b |
| Prism                 | Graphpad | Version 9.0 |
| Flow cytometer sorter | Sony | M900 |
| FlowJo                | BDBiosciences | N/A |
| **Other**             |        |            |
| 35-mm glass dish      | Fluorodish | fd35-100 |
| MC38-H2B-mCherry colon cancer cell lines | Center for Systems biology, Boston, MA | N/A |
| Fluoview FV1000 confocal microscope | Olympus | FV1000 |
| Vivaview epifluorescence microscope | Olympus | LCV110U |
| Dorsal skinfold chamber | Custom-built | N/A |
| Isoflurane            | Vetone, Fluriso | 26675-46-7 |
| **Deposited Data**    |        |            |
| Image and Signal Processing Code | GitHub / Zenodo | [https://doi.org/10.5281/zenodo.5668359](https://doi.org/10.5281/zenodo.5668359) |
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Kevin R. King (krking@ucsd.edu).

Materials availability
All unique/stable reagents generated in this study are available from the lead contact without restriction. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Dr. Kevin R. King (krking@ucsd.edu).

Data and code availability
- This paper does not report Standardized datatypes. All data reported in this paper will be shared by the lead contact upon request.
- All original code has been deposited at https://github.com/krking/GCaMP-Communication and is publicly available (https://doi.org/10.5281/zenodo.5688359).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
Mouse experiments were approved and conducted under the oversight of University of California San Diego Institutional Animal Care and Use Committee (#17144) or approved by the Subcommittee on Animal Research Care at Massachusetts General Hospital. All mice were maintained in a pathogen-free environment. Csf1rCreGCaMP5fl calcium reporter mice were created by breeding “Csf1r-Cre” C57CL/6-Tg(Csf1r-cre)1Mnz/J (The Jackson Laboratory; stock 029306), which expresses Cre recombinase under the regulation of the Csf1r promoter, with “GCaMP5” calcium reporter mice, B6;129S6-Polr2aIpo-CAG-GCaMP5g,-tdTomatoTvrd (The Jackson Laboratory; stock 024477). All Csf1r-expressing cells and their progeny express the GCaMP5 calcium reporter and constitutive tdTomato in a bicistronic fashion (41, 47). Genotyping was performed using Jackson Laboratory recommended methods. Experiments were performed with male reporter mice between 10-20 weeks of age.

METHOD DETAILS

Tissue processing
Peripheral blood for flow cytometric analysis was collected by retro-orbital bleeding using heparinized capillary tubes (BD Diagnostic Systems) and red blood cells were lysed with 1x red blood cell lysis buffer (BioLegend). For organ harvest, mice were perfused through the LV with 10 mL of ice-cold PBS. Hearts, spleen, lung and kidney were enzymatically digested for 1h under continuous agitation at 37°C in 450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNase I, and 60 U/ml hyaluronidase (Sigma) and filtered through a 40 μm nylon mesh in FACS buffer to generate a cell suspension for staining and flow cytometric analysis as previously described (48). To define the anatomical distribution of Csf1r-Cre-induced reporter cells within solid organs, we cut 1 mm sections with a tissue slicer (Zivic Instruments) and examined the spatial distribution of reporter fluorescence in each tissue using a Nikon STORM super resolution confocal microscope at UCSD.

Flow cytometry
Isolated cells were stained at 4°C in FACS buffer (PBS supplemented with 2.5% bovine serum albumin) with and hematopoietic lineage markers including Ly6G (BioLegend, clone 1A8, 1:600), CD11b (BioLegend, clone M1/70, 1:600) and Ter119 (BioLegend, clone TER-119, 1:600). This was followed by a second staining for NK1.1 (BioLegend, clone PK 136, 1:600), Thy1 (CD90.2, BioLegend, clone 53-2.1 1:600), and Ly6C (BioLegend, clone HK1.4 1:600). Cell suspensions were labeled with DAPI just prior to flow cytometric analysis to allow exclusion of dead cells. Doublets, erythrocytes, and dead cells were excluded by forward scatter, Dapi, and Ter119. Neutrophils were identified as (Ter119low/CD11bhigh/Ly6Ghigh). Monocytes were identified as (Ly6Glow/Ter119low/CD11bhigh/Ly6Chigh). NK or T cells were identified as (CD11blow/Ly6Glow/Nk1.1high or CD90.2high) respectively. The Cre-induced fraction of each hematopoietic lineage subset was determined based on the fraction that was tdTomatohigh. Data was acquired by Sony sorter MA900 at UCSD and analyzed with FlowJo software.

Cell culture
Bone marrow derived macrophages (BMDMs) from the Csf1rCreGCaMP5fl reporter mice were isolated, cultured in 10% FBS 1% Pen/Strep-containing DMEM, and differentiated with addition of 10 ng/mL recombinant m-CSF (Peprotech) (every other day media changes) for a period of 7 days as previously described. 10 μg of immunogenic HT DNA (Invivogen) was complexed with a
Lipofectamine transfection agent (ThermoFisher) in serum free-media and added to 1 million BMDMs in a 6-well multi-well plate with serum- and mCSF-containing media for each experiment.

Time-lapse imaging of calcium reporter dynamics in vitro and in vivo
Low temporal frequency imaging was performed using an Olympus Vivaview epifluorescence microscope with a sampling interval of 2 minutes. DIC/phase contrast and GFP fluorescence time lapse images were captured before and after stimulation with complexed immunogenic dsDNA. For high frequency time-lapse imaging, we used a Fluoview FV1000 confocal microscope at a scan speed of 2 and sampling interval of 0.5 seconds. Frame sizes are indicated by scale bars. For intravital imaging, dorsal window chambers were installed in adult male reporter mice (8 to 24 weeks old). After window stabilization, 1 million MC38-H2B-mCherry colon cancer cells were injected into the tissue underlying the window and imaging was performed at serial time points.

Quantitative analysis of epifluorescence imaging
Maximum intensity projections of fluorescence images were used to define regions of interest (ROIs) for each single cell. For each ROI, calcium fluorescence was quantified across time, normalized to median of fluorescence trace, and expressed as $\Delta F/F = (F[t] - F[median])/F[median]$. Cell viability was quantified based on microscale changes in differential DIC images defined as DIC[t] – DIC[t-1]. Pixels of each differential DIC image were summed, resulting in a scalar at each time point that reached a minimum that was interpreted as the timing of cell death. Cells were aligned by setting the timing of peak calcium fluorescence to zero.

Inference of cell communication from calcium reporter time lapse imaging
All analyses were performed using ImageJ/Fiji and MATLAB (Mathworks). Single cell regions of interest (ROI) were defined using ImageJ/Fiji and single cell fluorescence versus time was extracted for each ROI. Background was corrected using a rolling ball algorithm and average fluorescence was quantified for each ROI across time. Each ROI fluorescence time-series was smoothed using a zero-phase digital low pass infinite filter which strengthens the passband signals with a cutoff of 0.45 Hz half power frequency. Peak-finding was performed and transformed into unit magnitude impulses located at the time of each fluorescence peak and termed the time of calcium elevation ($T_{Ca}$). This allowed construction of a discrete time series calcium elevation train $S_i[t]$ for each cell $i$. Cross correlation plots were generated by rank ordering all calcium elevations of all cells within an individual movie. For each pair of calcium elevation pairs, we calculated the product of the difference in calcium elevation timing $\Delta T_{Ca}$ and the Euclidean distance weighted by raising to the 1.5 power ($D^{1.5}$).

Spatiotemporal excess synchrony pipeline details
Methods for temporal excess synchrony calculation are detailed in the primary manuscript text. Spatiotemporal synchrony was calculated as $(\Delta S/w)$ for each 50 µm x 50 µm sub-image, containing an average of 11 active ROIs, at moving temporal windows of size $w$. Regions of excess synchrony that were connected in either time or space were combined and interpreted as a single cell communication event. Synchrony “stringency” was defined a margin above $S_{th}$ that would be interpreted as a genuine cell communication. It was expressed as a fraction of $S_{max}-S_{th}$ for each movie and therefore ranged between 0 and 1.

QUANTIFICATION AND STATISTICAL ANALYSIS
All statistical analyses were conducted with GraphPad Prism software and MATLAB. Data are presented as mean ± SEM. Statistical significance was evaluated using the two-sided Mann-Whitney test or Kolmogorov-Smirnov test. $p$ values less than 0.05 were considered to denote significance.