Accurate quantification of astrocyte and neurotransmitter fluorescence dynamics for single-cell and population-level physiology

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Recent work examining astrocytic physiology centers on fluorescence imaging, due to development of sensitive fluorescent indicators and observation of spatiotemporally complex calcium activity. However, the field remains hindered in characterizing these dynamics, both within single cells and at the population level, because of the insufficiency of current region-of-interest-based approaches to describe activity that is often spatially unixed, size-varying and propagative. Here we present an analytical framework that releases astrocyte biologists from region-of-interest-based tools. The Astrocyte Quantitative Analysis (AQuA) software takes an event-based perspective to model and accurately quantify complex calcium and neurotransmitter activity in fluorescence imaging datasets. We apply AQuA to a range of ex vivo and in vivo imaging data and use physiologically relevant parameters to comprehensively describe the data. Since AQuA is data-driven and based on machine learning principles, it can be applied across model organisms, fluorescent indicators, experimental modes, and imaging resolutions and speeds, enabling researchers to elucidate fundamental neural physiology.

With increased prevalence of multiphoton imaging and optical probes to study astrocyte physiology¹–⁷, many groups now have tools to study fundamental processes that previously remained unclear. Recent work has focused on new ways to decipher how astrocytes respond to neurotransmitter and neuromodulator circuit signals¹–⁷ and how the spatiotemporal patterns of their activity shape local neuronal activity⁸–¹⁰. Recording astrocytic dynamics to decode their disparate roles in neural circuits has centered on expression of genetically encoded probes to carry out intracellular calcium (Ca²⁺) imaging using GCaMP variants⁹. In addition, many groups study astrocytic function by performing extracellular glutamate imaging using GluSnFR² and several more recently developed genetically encoded fluorescent probes for neurotransmitters such as GABA⁴, norepinephrine (NE)⁵, ATP⁶ and dopamine⁷ are poised to further expand our understanding of astrocytic circuit biology.

Compared with neuronal Ca²⁺ imaging, astrocytic GCaMP imaging presents particular challenges for analysis due to the complex spatiotemporal dynamics observed. Astrocyte-specific analysis software programs have been developed to capture these Ca²⁺ dynamics, several of which identify subcellular regions-of-interest (ROIs) for analysis¹³. Likewise, GluSnFR imaging analysis techniques are based on manually or semimanually selected ROIs, or on analysis of the entire imaging field together as one ROI¹²,¹⁶,¹⁸. Thus, most, although not all¹²,¹⁸, other current techniques rely on the conceptual framework of ROIs for image analysis. However, astrocytic Ca²⁺ and GluSnFR fluorescence dynamics are particularly ill-suited for ROI-based approaches, because the concept of the ROI has several inherent assumptions that cannot be satisfied for astrocytic activity data. For example, astrocytic Ca²⁺ signals can occupy regions that change size or location across time, propagate within or across cells and spatially overlap with other Ca²⁺ signals that are temporally distinct. ROI-based approaches assume that for a given ROI, all signals have a fixed size and shape, and all locations within the ROI undergo the same dynamics, without propagation. Accordingly, ROI-based techniques may over- or under-sample these data, obscuring true dynamics and hindering discovery. An ideal imaging analysis framework for astrocytes would take into account all of these dynamic features and be free of ROI-based analytical restrictions. In addition, an ideal tool should be applicable to astrocyte imaging data across spatial scales, encompassing subcellular, cellular and population-wide fluorescence dynamics.

We set out to design an image analysis toolbox that would capture the complex, wide-ranging fluorescent signals observed in most dynamic astrocyte imaging datasets. We reasoned that a non-ROI-based approach would better describe the observed fluorescence dynamics, and applied probability theory, machine learning and computational optimization techniques to generate an algorithm to do so. We name this resulting software package AQuA and validate its utility by applying it to simulated datasets that reflect the specific features that make analyzing astrocyte data challenging. We next apply AQuA to experimental two-photon imaging data—ex vivo Ca²⁺ imaging of GCaMP6 from acute cortical slices; in vivo Ca²⁺ imaging of GCaMP6 in primary visual cortex (V1) of awake, head-fixed mice; and ex vivo extracellular glutamate, GABA and NE imaging. In these test cases, we find that AQuA accurately detects fluorescence dynamics by capturing events as they change in space and time, rather than from a single location, as in ROI-based approaches. AQuA outputs a comprehensive set of biologically relevant parameters from these datasets, including propagation speed.

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propagation direction, area, shape and spatial frequency. Using these detected events and associated output features, we uncover neurobiological phenomena.

A wide variety of functions have been ascribed to astrocytes, and a key question currently under examination in the field is whether certain types of Ca\textsuperscript{2+} activities correspond to particular neurobiological functions. However, current techniques with which to classify these observed dynamics remain inadequate since they do not capture many of the dynamics recorded in fluorescent imaging of astrocytic activity. The framework we describe allows for a rigorous, in-depth dissection of astrocyte physiology across spatial and temporal imaging scales, and sets the stage for a comprehensive categorization of heterogeneous astrocyte activities both at baseline and after experimental manipulations.

Results

Design principles of the AQuA algorithm. To move away from ROI-based analysis approaches and accurately capture heterogeneous astrocyte fluorescence dynamics, we designed an algorithm to decompose raw dynamic astrocyte imaging data into a set of quantifiable events (Fig. 1a, Supplementary Video 1 and Supplementary Figs. 1–3). Here, we define an event as a cycle of a signal increase and decrease that coherently occurs in a spatially connected region defined by the fluorescence dynamics, not a priori by the user or the cell morphology. Algorithmically, this definition is converted to the following two rules: (1) the temporal trajectory for an event contains only one peak (single-cycle rule; Fig. 1b), and (2) adjacent locations in the same event have similar trajectories (smoothness rule; Fig. 1b).

The task of the AQuA algorithm is to detect all events, and, for each event, to identify the temporal trajectory, the spatial footprint and the signal propagation. Briefly, our strategy of event detection is to (1) explore the single-cycle rule to find peaks, which are used to specify the time window and temporal trajectory; (2) explore the smoothness rule to group spatially adjacent peaks, whose locations specify the footprint; (3) apply machine learning and optimization techniques to iteratively refine the spatial and temporal properties of the event to best fit the data; and (4) apply statistical theory to determine whether a detected event is purely due to noise (Fig. 1).

Full statistical and computational details are provided in the Methods, but we highlight one technical innovation (graphical time warping (GTW))\textsuperscript{19} and one concept (the single-source rule) here that jointly enable a nuanced analysis of astrocyte fluorescence dynamics when applied to experimental datasets. To the best of our knowledge, signal propagation has never been rigorously accounted for and has been considered an obstacle to analysis. With GTW, we can estimate and quantify propagation patterns in the data. With the introduction of the single-source rule (Fig. 1b), each event only contains a single initiation source and we can separate events that are initiated at different locations but meet in the middle. The single-source rule also allows us to divide large-scale activity across an entire field of view into individual events, each with a single initiation location.

The output of the event-based AQuA algorithm is a list of detected events, each associated with three categories of parameters: (1) the spatial map indicating where the event occurs, (2) the dynamic curve corresponding to fluorescence change over time (dF/F) and (3) the propagation map indicating signal propagation. For each event, we use the spatial map to compute the event area, diameter and shape of the domain it occupies (Fig. 1c). Using the dynamic curve, we can calculate maximum dF/F, duration, onset-time, rise-time and decay-time. Using the propagation map, we extract event initiation location, as well as propagation path, direction and speed. In addition, AQuA computes features involving more than one event, such as the frequency of events at a position, and the overall number of events in a specified region or cell. A complete list of features is in the Methods.

Validation of AQuA using simulated data. To validate AQuA, we designed three simulation datasets to know the ground truth dynamics of each event. These datasets independently vary the three key phenomena in astrocyte imaging datasets that cause ROI-based approaches to misanalyze the data: size variability, location variability and propagation. While these phenomena usually co-occur in real datasets, we simulated each independently to examine its individual impact and test AQuA’s performance relative to other image analysis tools, including CalimAn\textsuperscript{20}, Suite2P\textsuperscript{21}, CaSCaDe\textsuperscript{22} and GECI-quant\textsuperscript{23}. CalimAn and Suite2P are widely used for neuronal Ca\textsuperscript{2+} imaging analysis while CaSCaDe and GECI-quant were designed specifically for Ca\textsuperscript{2+} activity in astrocytes; all four methods are ROI-based. In our analysis of these simulated datasets, we optimally tuned the ROI-detection for all methods for an objective comparison of the best performance of each method. We also systematically changed the signal-to-noise ratio (SNR) to examine the effect of noise.

To evaluate the performance on all simulated datasets, we used two measures: intersection over union (IoU) and a map of the event counts. IoU measures the consistency between the detected and ground-truth events, and takes into account both the spatial and temporal accuracy of detected events. IoU ranges from 0 to 1, where 1 indicates perfect detection and 0 indicates a complete failure in detection. The map of the event counts is obtained by counting the number of events at each pixel in the field, and is used to visually assess the accuracy of event detection results by a comparison with the ground-truth map.

We first studied the impact of size-varying events (Fig. 2a), in which multiple events occurred at the same location and the event centers remained fixed, but sizes changed across different events. The degree of size change is quantified using size-change odds (see Methods) where a size-change odds of 1 indicates events with the same size, while an odds of 5 is the largest simulated size change. For example, when the odds are at 5, events with sizes randomly distributed between 0.2 and 5 times the baseline size are simulated. When there was no size change (odds = 1), all methods performed well with IoUs near 0.95 (Fig. 2a). When the size change was increased, AQuA still performed well (IoU = 0.95), while all other methods quickly dropped to 0.4–0.5. We next studied the impact of SNR on performance by varying SNR, but fixing the size-change odds. AQuA performed better with increasing SNR and achieved nearly perfect detection accuracy (IoU = 1) at 20 dB. In comparison, all other methods had an IoU less than 0.6, even at high SNR (Fig. 2a). We also examined the results by visualizing event counts at each pixel (Supplementary Figs. 4 and 5). These maps show that AQuA faithfully reported the events under various SNRs but the other methods had erroneous event counts with artificial patterns.

We next focused on shifting event locations. In these simulated datasets, event size was fixed but location changed, the degree of which was represented by a location-change score (Fig. 2b). Zero indicates no location change and greater values represent larger degrees of change. Here, results are similar to changing size, as above. AQuA models the location change well and its performance is not affected by degree of location change. Likewise, AQuA reached near perfect results when SNR was high. In contrast, all other analysis methods performed poorly with changing locations (Fig. 2b and Supplementary Fig. 4).

In our third simulated dataset, we asked how fluorescence signal propagation impacts the performance of AQuA and other methods. Two propagation types—growing and moving—were simulated in this dataset (Fig. 2c), although they were also separately evaluated (Supplementary Fig. 6). Propagation frame number denotes the difference between the earliest and latest onset times within a single event. When propagation frame number is zero, all signals within one ROI are synchronized and there is no propagation. Similar results to the two scenarios above were obtained here, with AQuA...
out-performing all of the other methods by a large margin. These results indicate that AQuA handles various types of propagation well, while the performance of other methods degrades rapidly when propagation is introduced.

In summary, when any of the three ROI-violating factors—size variability, location variability and propagation—is introduced, other methods do not accurately capture the dynamics of the simulated data, and AQuA outperforms them by a large margin. We expect that the performance margin on real experimental data is larger than those quantified in the simulation studies here, since real data exhibit multiple ROI-violating factors. However, these IoU analyses and the event count visualizations informed us about different types of errors observed in ROI-based methods: CaSCaDe tends to over-segment, as it is based on watershed segmentation, and GECI-quant is particularly challenged by noise, causing many lost signals (Supplementary Fig. 5). We note that propagation caused ROI-based approaches to quickly decline in performance, with GECI-quant influenced by noise level and CaSCaDe's assumption of synchronized signals not allowing for accurate capture of event dynamics.

AQuA enables identification of single-cell physiological heterogeneities. To test AQuA's performance on real astrocyte fluorescence imaging data and ask whether AQuA could classify Ca^{2+} activities observed in single cells, we next ran AQuA on Ca^{2+} activity recorded from astrocytes in acute cortical slices from mouse V1 using two-photon microscopy. We used a viral approach to express the genetically encoded Ca^{2+} indicator GCaMP6f in layer 2/3 astrocytes. Unlike ROI-based approaches, AQuA detects both propagative and nonpropagative activity, revealing Ca^{2+} events with a variety

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**Fig. 1 | AQuA-based event detection.**

**a**, Individual representative frames from 5-min ex vivo astrocytic GCaMP imaging experiment (top; Supplementary Video 1) with AQuA-detected events shown below. Each color represents an individual event, and colors are chosen at random. The right column shows the average GCaMP fluorescence (top) and all AQuA-detected events (bottom) from the entire video. Note that contrast differs between rows to highlight events. Similar event detection results are observed in all 48 ex vivo, 46 in vivo GCaMP datasets and 14 GluSnFR datasets used in this report. **b**, Flowchart of AQuA algorithm. Raw data are presented as a stack of images across time with gray level indicating signal intensity. In the 'detect peaks' panel, five peaks are detected and highlighted by solid diamonds, each color denoting one peak. Based on the single-cycle rule and spatial adjacency of the apexes (solid dots) of each peak, peaks are clustered into spatially disconnected groups. Based on the smoothness rule, propagation patterns are estimated for each peak group. By applying the single-source rule, two events are detected for peak group 1. Three total events are detected. **c**, Feature extraction. Based on the event detection results, AQuA outputs four sets of features relevant to astrocytic activity: (1) propagation-related (path, direction and speed); (2) source of events, indicating where an event is initiated; (3) features related to the event footprint, including area and shape (event 1 is plotted here); and (4) features derived from the dF/F dynamics.
of shapes and sizes (Fig. 3a, left). Further, since AQuA detects Ca\textsuperscript{2+} events’ spatial footprint and time-course, we can apply AQuA to measure the propagation direction each event travels in over its lifetime. Imaging single cells, we used the soma as a landmark, and classified events as traveling toward the soma (pink), away from the soma (purple) or static (blue) for the majority of its lifetime (Fig. 3a, right). We then combined multiple measurements (size, propagation direction, duration and minimum proximity to soma) into one spatiotemporal summary plot (Fig. 3b). Since astrocytes exhibit a wide diversity of Ca\textsuperscript{2+} activities across subcellular compartments\cite{6,22,23}, plotting the signals this way rather than standard dF/F transients highlights these heterogeneities, allows us to map the subcellular location of the Ca\textsuperscript{2+} signals and enables a quick, visual impression of large amounts of complex data (Supplementary Fig. 7). We note that while the expression of GCaMP6 in these experiments enabled us to analyze events within single cells, some probes do not allow clear delineation of single cells. However, a secondary fluorophore (such as TdTomato) often serves the purpose of defining the morphology of single cells, and the AQuA software is designed to overlay morphological masks on the dynamic fluorescence channel.

We next asked whether some subcellular regions of astrocytes have more dynamic activity than others. Although we detected more static events than dynamic ones overall (Supplementary Fig. 8a), we observed a higher proportion of dynamic events than static events in the soma (59%; Fig. 3c and Supplementary Fig. 8b). We then characterized events by propagation direction and event initiation location (Fig. 3d). Events that begin close to the soma and propagate away (purple) were on average larger than the events propagating toward the soma (pink; two-tailed t-test). Similarly, those events that began close to the soma and propagated away showed a longer duration than events propagating toward the soma (two-tailed t-test; Fig. 3e and Supplementary Fig. 8).

AQuA automatically extracts many features that can be used to form a comprehensive Ca\textsuperscript{2+} measurement matrix, where each row represents an event and each column an extracted feature (Supplementary Fig. 9). Dimensionality reduction applied to this matrix can then be used to visualize each cell's Ca\textsuperscript{2+} signature (Supplementary Fig. 9, white rows separate individual cells). To do this, we applied t-distributed stochastic neighbor embedding (t-SNE)\cite{24}, followed by k-means clustering to assign the cells to groups (Supplementary Fig. 9), revealing clusters marked by cells with large differences in median frequency (Fig. 3f). Astrocytic Ca\textsuperscript{2+} frequency is commonly measured as the number of transients in time within an ROI. Here, we instead define frequency from an event-based perspective in two ways: (1) for each event, the number of other events that overlap in time; and (2) for each event, the number of other events that overlap in space. We used these two measures (temporal and spatial overlap) and several other extracted measures (Supplementary Fig. 9) to construct the matrix used for t-SNE visualization and clustering. We then tested how well our AQuA-specific features perform at clustering compared with two ROI-based methods (Fig. 3g), and found that the AQuA-based method outperformed the others. In fact, even when we only use AQuA-specific features—area, temporal overlap, spatial overlap and propagation speed—for this analysis and remove all features that can be extracted from ROI-based methods, AQuA still significantly outperforms in clustering cells (Supplementary Fig. 9g–i). AQuA-extracted features that correspond to those that can be obtained by ROI-based methods—frequency, amplitude, duration—do not allow clustering significantly better than the ROI-based approaches themselves (Supplementary Fig. 9g–i), suggesting that AQuA-specific features best capture dynamic fluorescence features that vary among single cells. This indicates that AQuA may be used to extract data from existing ex vivo Ca\textsuperscript{2+} imaging datasets to reveal previously uncovered dynamics and sort cells into functionally relevant clusters.
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2.11 × Previous studies have described temporal details of astrocyte activity using GCaMP6f imaging, yet have left largely unaddressed the combined spatiotemporal properties of Ca$^{2+}$ activity across multiple cells. Here, we explored whether AQuA can uncover spatial patterns within populations of cortical astrocytes in an awake animal, and carried out head-fixed, two-photon imaging of GCaMP6f in V1 astrocytes. In vivo astrocytic Ca$^{2+}$ events are small, focal, desynchronized Ca$^{2+}$ activities, and large, coordinated activities that we refer to as bursts. Importantly, AQuA detected both types of Ca$^{2+}$ activity within the same in vivo imaging datasets (Supplementary Video 2 and Fig. 4a). Similar to previous studies, we observed many of the bursts co-occurring with locomotion (Fig. 4b, pink), and many events within these bursts displayed propagation (Fig. 4c, top). Propagative events were larger in area and propagated greater distances than those occurring during the inter-burst periods (Fig. 4c, bottom). To test whether AQuA could help us discover discrete features of this phenomenon, we next focused our investigation on the burst-period events (Supplementary Fig. 10).

Fig. 3 | AQuA features capture heterogeneities among single astrocytes. a, Representative GCaMP6f ex vivo image (left) with AQuA events overlaid from 1 min of a 5 min video. Soma marked with black ‘s’ (Supplementary Video 1). Right: representative image sequence for each propagation direction class (blue, static; pink, toward soma; purple, away from soma). Data from a total of 11 cells from five slices. b, Spatiotemporal plot of Ca$^{2+}$ activity from 1 min of video. Each event is represented by a polygon that is proportional to its area as it changes over its lifetime. c, Distribution of dynamic and static events as a function of minimum distance from soma. All bin widths calculated by Freedman–Diaconis’s rule. d, Left: propagative event size versus starting distance from soma, segregated by propagation direction. Dashed gray line denotes half the distance to the soma and white arrow. Mean area (** P < 0.001). Right: event colors indicate event number per min (0.2–4) at each location. Median (red) and interquartile range (gray) from cells in each cluster in Supplementary Fig. 9 (one-tailed Wilcoxon rank-sum test, *** P < 0.001). g, Centroid distances between cells from two clusters determined by t-SNE plots of Ca$^{2+}$ activity using features calculated from ROIs and 5 × 5 μm$^2$ tiles (left) (right, one-tailed paired t-test, *** P = 9.37 × 10$^{-6}$ (tiles), 2.11 × 10$^{-6}$ (ROIs)).

In vivo astrocytic Ca$^{2+}$ bursts display anatomical directionality. Recent interest in astrocytic activity at the mesoscale has been driven by multi-cellular astrocytic Ca$^{2+}$ imaging, yet have left largely unaddressed the combined spatiotemporal properties of Ca$^{2+}$ activity at the circuit-level, across multiple cells. Here, we explored whether AQuA can uncover spatial patterns within populations of cortical astrocytes in an awake animal, and carried out head-fixed, two-photon imaging of GCaMP6f in V1 astrocytes. In vivo cortical astrocytes exhibit both small, focal, desynchronized Ca$^{2+}$ activities, and large, coordinated activities that we refer to as bursts. Importantly, AQuA detected both types of Ca$^{2+}$ activity within the same in vivo imaging datasets (Supplementary Video 2 and Fig. 4a). Similar to previous studies, we observed many of the bursts co-occurring with locomotion (Fig. 4b, pink), and many events within these bursts displayed propagation (Fig. 4c, top). Propagative events were larger in area and propagated greater distances than those occurring during the inter-burst periods (Fig. 4c, bottom). To test whether AQuA could help us discover discrete features of this phenomenon, we next focused our investigation on the burst-period events (Supplementary Fig. 10).

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To analyze the structure of these burst-period Ca\(^{2+}\) events, we investigated fluorescence propagation across multiple spatial scales: at the levels of individual events, of subregions of the imaging field encompassing multiple events and of the entire imaging field. At the level of individual events within a single burst, plotting the individual event direction within the entire field of view did not reveal a consistent propagation direction (Fig. 4d). However, when we divided our field of view into equivalently sized, subregional tiles (Fig. 4e),...
we observed more consistent propagation direction within single subregions (Fig. 4f). When we plot the cumulative count of the percentage of bursts with regions that propagate in the same direction, we indeed observe that this curve is right-shifted compared with a simulated random assignment of majority regional propagation direction (Fig. 4g), suggesting regularity in the propagation pattern within bursts that only becomes apparent at larger spatial scales. Thus, we next explored whole imaging field dynamics during Ca^2+ bursts. The percentage of the active field of view varied across burst periods (Fig. 4b), with a wide variability from few to hundreds of events (Fig. 4h). To control for number and size of events, we used the difference between each event's onset time to calculate a single-burst-wide propagation direction (Fig. 4h, black arrow). Doing so revealed a consistent posterior-medial directionality of population Ca^2+ activity (Fig. 4i). Although Ca^2+ bursts have been previously observed using GCaMP6 imaging in awake mice^{19}, consistent spatial directionality with respect to the underlying anatomy has never been described. This observed posterior-medial directionality may be revealing anatomical and physiological underpinnings of these bursts, and since they have been shown to be at least partly mediated by NE^{25}, they could be reflective of responses to incoming adrenergic axons originating in the locus coeruleus. Regardless of mechanism(s), these results suggest that in vivo, astrocytic Ca^2+ propagation dynamics differ depending on the spatial scale examined, which may explain previously described discrepancies.

AQuA-based analysis of extracellular neurotransmitter fluorescence dynamics. We next asked whether AQuA could be used...
to detect other spatiotemporally complex fluorescent dynamics distinct from astrocytic Ca\(^{2+}\). We decided to image extracellular-facing neurotransmitter probes, including GluSnFR\(^{10,32}\), since astrocytes regulate extracellular glutamate. GluSnFR dynamics are much faster than GCaMP dynamics, causing detection to be very susceptible to low SNR. This can be an additional challenge and many previous GluSnFR analyses have relied on averaging across multiple trials. While GluSnFR has been expressed both in astrocytes and in neurons previously\(^{2,3,5,6}\), how cell type-specific expression and morphology determines its fluorescent dynamics has not been fully explored\(^{2,3}\). Likewise, no previously applied analytical tools have been reported to automatically detect GluSnFR or other neurotransmitter events to accommodate different event sizes and shapes. Here, we explored whether application of AQuA could be used to detect cell type-specific differences in glutamate dynamics that may be based on heterogeneous underlying morphologies and cell biological mechanisms.

We expressed GluSnFR in either astrocytes or neurons using cell type-specific viruses\(^{3}\) and carried out two-photon imaging of spontaneous GluSnFR activity in acute cortical V1 slices. Distinct morphological differences between astrocytic and neuronal expression of GluSnFR were evident, as observed previously\(^{2,3,5,6}\) (Fig. 5a). We applied AQuA to these datasets to detect significant fluorescent increases, and were able to detect events that were too small and fast to be detected by eye (Supplementary Video 3). AQuA-detected events were confirmed by post hoc ROI-based analysis. We found that 62% of astrocytic events had an area less than the size of a single astrocyte, and 8% of astrocytic and 35% of neuronal glutamate events had a small maximum dF/dt (less than 0.5). Because GluSnFR events have previously been detected by spatial averaging or by manual detection, many AQuA-detected events are most likely missed by ROI-based methods\(^{5,6}\) (Supplementary Fig. 11). Because AQuA detects events independently from shape or size, events of heterogeneous size and shape were revealed during this analysis (Fig. 5a,b). A large proportion of these spontaneous GluSnFR events changed size over the course of the event, with 42% of total astrocytic and 32% of total neuronal glutamate events exhibiting area changes. On average, astrocytic GluSnFR events were significantly larger (274 ± 39.56 μm\(^2\)) than neuronal events (172 ± 57.06 μm\(^2\)), sometimes encompassing an entire astrocyte (Supplementary Fig. 11). Neuronal GluSnFR events were significantly more circular (Fig. 5b–d), perhaps reflecting morphological differences between cell types. Between cell types, GluSnFR events also exhibited different size dynamics (Fig. 5b,c). We also observed that the rate of size decrease of astrocytic events between frames was larger than that of neuronal events (Fig. 5c), which may reflect differential synaptic and extrasynaptic glutamate dynamics in proximity to subcellular compartments of each cell type.

After showing that AQuA-based detection is effective for quantification of spontaneous GluSnFR activity, we tested its performance on fast, evoked glutamate events, since GluSnFR is used to measure synaptic glutamate release at fast acquisition rates\(^{2,3}\). To do this, we performed fast (~100 Hz) GluSnFR imaging while photocaptivating a caged glutamate compound (RuBi-glutamate\(^{10,32}\)) with a second laser beam. In these experiments, we uncaged glutamate for varying durations (25–150 ms), and applied AQuA to detect these small-scale, fast events (Fig. 5d, right). AQUA detection showed high accuracy levels across all uncaging durations, with a minimum of 96% average accuracy across durations (Fig. 5d, right; n = 5 cells, three replicates per cell), indicating that AQuA works well for event detection at fast frame rates.

We tested whether AQuA could be used with other probes relevant for astrocyte-neuron physiology by imaging two recently developed genetically encoded probes that report extracellular neurotransmitter dynamics: GABASnFR\(^{3}\) and GRAB-NE\(^{12}\). We expressed GABASnFR (Fig. 5e, left) in cortical astrocytes and GRAB-NE in cortical neurons (Fig. 5f, left), and performed ex vivo imaging with bath application of either GABA or NE. For GABASnFR expression in individual astrocytes, AQUA-detected events increased in amplitude and area with cell-specific dynamics (Fig. 5e, right). The widespread neuronal expression of GRAB-NE allowed us to detect the location, amplitude and area of NE waves as they progressed across the slice (Fig. 5f, right), indicating that AQUA may be useful to quantify propagating wavefronts in other contexts. Together, these results suggest that AQUA-based detection can be used to quantify dynamics of extracellular molecules at a range of speeds and spatial spreads, across multiple cell types and expression patterns.

**Discussion**

With the development of an event-based analysis tool, we have enabled accurate quantification of fluorescence dynamics that are unfixed, propagative and size-varying. Here, we demonstrate that AQUA performs better than other image analysis methods on simulated datasets, and describe event detection using several genetically encoded indicators. AQUA can also be applied to datasets not directly tested here, including those captured under different magnifications and spatial resolutions, as well as under confocal or wide-field imaging. Since AQUA functions independently from frame rate, datasets captured faster or slower\(^{17,25}\) are also amenable to an AQUA-based analysis. Further, AQUA is applicable to fluorescence indicators other than the ones tested here, particularly those that exhibit complex dynamics.

We envision the AQUA software as an enabling problem-solving tool for a wide range of astrocyte physiological questions, because it accurately captures dynamics exhibited by commonly used fluorescent indicators. Since AQUA-specific features captured observed heterogeneities among single cells, these features may be more physiologically relevant than the standard measurements (amplitude, frequency, duration) used to describe astrocytic physiology. Beyond baseline differences, we expect that AQUA will be a powerful tool to quantify physiological effects of pharmacological, genetic and optogenetic manipulations.

Significant disagreement about basic physiological functions of astrocytes remains. One outstanding issue is whether astrocytes release transmitters such as glutamate. While we do not address this topic here, we expect that the heterogeneous activities uncovered using in-depth GluSnFR analysis may be key in determining different sources of glutamate under different conditions, and could help to untangle conflicting data. AQUA here may be particularly useful in this regard as next-generation GluSnFR variants become available and make multiplexed imaging experiments increasingly accessible\(^{11}\). Astrocyte Ca\(^{2+}\) imaging data can largely be grouped into two categories: single-cell imaging and population-wide imaging focusing on many cells. Experimental data and neurobiological conclusions from these two groups can differ quite widely. This may be due, in part, to population-wide bursts observed with locomotion onset in vivo. Many ROI-based techniques used to analyze these bursts can under-sample interburst events by swamp ing out smaller or shorter signals. Our technique can sample small- and large-scale activity in the same dataset and may aid researchers in resolving outstanding physiological problems.

As demonstrated by its utility with Ca\(^{2+}\), glutamate, GABA and NE datasets, AQUA can now be applied to many other fluorescence imaging datasets that exhibit nonstatic or propagative activity, particularly since it is open-source and user-tunable. For example, recently described Ca\(^{2+}\) dynamics in oligodendrocytes display some similar properties to astrocyte Ca\(^{2+}\) (refs.\(^{33,34}\)). Likewise, subcellular neuronal compartments, such as dendrites or dendritic spines, can exhibit propagative, wave-like Ca\(^{2+}\) signals\(^{35}\) while whole-brain neuronal imaging can capture burst-like, population-wide events\(^{36}\).

We predict that the potential applications of AQUA are wide, but AQUA also has limitations. Since it detects local fluorescence...
increases, AQUA is not well suited to analyze morphological dynamics, such as those observed in microglia, and it does not improve on tools built for analyzing somatic neuronal Ca$^{2+}$ (refs. 21,22), where ROI assumptions are well satisfied. In addition, AQUA was optimized for two-dimensional (2D) datasets, as these comprise the majority of current astrocyte imaging experiments. As techniques for volumetric imaging advance, an extension to accommodate three-dimensional (3D) imaging experiments will be necessary. AQUA is expandable to 3D datasets since the algorithmic design is not restricted to 2D assumptions. A full 3D AQUA version is beyond the scope of this paper, but a 3D prototype performed well on simulated 3D data based on published astrocytes (Supplementary Fig. 12). These results suggest that a full 3D version will work on real volumetric datasets in the future and demonstrate that AQUA is a flexible and robust platform that can accommodate new data types without large changes to the underlying algorithm.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41593-019-0492-2.

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Methods

Viral injections and surgical procedures. All procedures were carried out in accordance with protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee. For slice experiments, neonatal mice (Swiss Webster, postnatal day (P) 0–4) were anaesthetized by crushed ice anesthesia for 3 min and injected with 90 nl total virus of AAV5-GFAP-ABC1D.Lck-GCaMP6f, AAV5-GFAP-ABC1D.cyo-GCaMP6f, AAV1-GFAP-GluSnFR, AAV1-hynx-GluSnFR, AAV2-GFAP-GABAinFR.F102G and AAV9-hynx-NE21 at a rate of 2–3 nl s⁻¹. Six injections 0.5 µm apart in a 2 × 3 grid pattern with 15 nl per injection into assumed V1 were performed 0.2 mm below pial surface using a UMP-3 microsyringe pump (World Precision Instruments). Mice were used for slice imaging experiments at P10–23.

For in vivo experiments, adult mice (C57BL/6, P50–P100) were given dexamethasone (5 mg kg⁻¹) subcutaneously before surgery and then anesthetized under isoflurane. A titanium headplate was attached to the skull using C&B Metabond (Parkell) and a 3-mm-diameter craniotomy was cut over the right hemisphere for gaining access to visual cortex. Two 300 nl injections (6000 nl total) of virus (AA V5-GFAP-ABC1D.cyo-GCaMP6f) were made into visual cortex (0.5–1.0 mm anterior and 1.75–2.5 mm lateral of bregma) at a depth of 0.2–0.3 mm and 0.5 mm from the pial surface, respectively. Virus was injected at a rate of 2 nl s⁻¹, with a 10 min wait following each injection to allow for diffusion. Following viral injection, a glass cranial window was implanted to allow for chronic imaging and secured using C&B metabond. Mice were given at least 10 days to recover, followed by habituation for 3 d to head fixation on a circular treadmill, before imaging.

Two-photon imaging. All two-photon imaging experiments were carried out on a microscope (Olympus Fluoview FV1000) equipped with two Ti:Sapphire lasers (SpectraPhysics). The laser beam was intensity-modulated using a Pockels cell (Conoptics) and scanned with linear or resonant galvanometers. Images were acquired with a x16, 0.8 numerical aperture (Nikon, in vivo GCaMP and ex vivo GRAB-NE) or x40, 0.8 numerical aperture objective (Nikon, ex vivo GCaMP, GluSnFR and GABAinFR) via a photomultiplier tube (Hamamatsu) using FluorView (Bruker) software. For imaging, 980 nm (GCaMP), 910 nm (GluSnFR and GABAinFR) or 920 nm (GRAB-NE) excitation and a 515/30 emission filter were used.

Ex vivo imaging. Coronal, acute neocortical slices (400 µm thick) from P10–P23 mice were cut with a vibratome (VT 1200, Leica) in ice-cold cutting solution (in mM): 27 NaHCO₃, 1.5 NaH₂PO₄, 220 sucrose, 2.6 KCl, 2 MgSO₄, 2 CaCl₂. Slices were incubated in standard continuously aerated (95% O₂/5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM): 123 NaCl, 26 NaHCO₃, 1 NaH₂PO₄, 10 dextrose, 3 KCl, 2 CaCl₂, 2 MgSO₄, heated to 37°C and removed from water bath immediately before introducing slices. Slices were kept in ACSF at room temperature until imaging. Experiments were performed in continuously aerated, standard ACSF. Two-photon scanning for all probes was carried out at 512 × 512-pixel resolution. Acquisition frame rates were 1.1 Hz (GCaMP), 4–100 Hz (GluSnFR) and 4–14 Hz (GRAB-NE). For GluSnFR imaging and RuBi-glutamate uncaging experiments, GluSnFR imaging was performed at 990 nm excitation to ensure that no RuBi-glutamate was released during scanning. Acquisition rates were 95–100 Hz, using resonant galvanometers. Next, 300 µM RuBi-glutamate was added to the circulating ACSF and, using a second MaiTai laser tuned to 800 nm, five uncaging points were successively uncaged at each cell at durations indicated in the figure and at power durations indicated in the figure and at power 3 mW, which were shown in control experiments. In vivo GCaMP imaging. At least 2 weeks following surgery, mice were head-fixed to a circular treadmill and astrocyte Ca²⁺ activity was visualized at ~2 Hz effective frame rate from layers 2/3 of visual cortex with a 512 × 512-pixel resolution at 0.8 µm per pixel. Locomotion speed was monitored using an optoswitch (Newark Element 14) connected to an Arduino.

AQUA algorithm and event detection. Overview of the AQUA algorithm. Astrocytic events are heterogeneous and varying with respect to many aspects of their formation. In our experiments, we attempt to model the temporal and spatial properties of astrocytic events so that our approaches can be applied to optimally extract fluorescent signals from background fluctuations. Here, we delineate the eight major steps in AQUA (Supplementary Fig. 1), discuss motivations behind the algorithm design and describe key technical considerations.

Step 1 (data normalization and preprocessing) removes experimental artifacts such as motion effects, and processes the data so that noise can be well approximated by a standard Gaussian distribution. Additional detail is paid to the variance stabilization, estimate of baseline fluorescence and variance. Step 2 (detecting active voxels) identifies for seed detection and Step 3 (detecting active voxels and their spatialtemporal patterns to achieve peak detection. To detect peaks, we start from a seed, which is modeled as a spatiotemporal local maximum. However, since random fluctuations due to background noise can also result in local maxima, we need to detect active voxels such that only the local maxima on the active voxels are considered seeds.

Here, active voxels are those likely to have signals. Step 5 involves clustering peaks to identify candidate peaks. Step 6 involves temporal filtering to smooth the data to reduce the impact of noise. Then, we calculate the z-scores for each voxel in the smoothed data. Here, z-score is computed as the value of the voxel divided by its standard deviation, which can be estimated as in the normalization procedure above, but now on the smoothed data. All voxels that have z-scores larger than a given threshold are considered tentative active voxels. A liberal threshold is used here to retain most signals, often at a z-score of 3. We next calculate the size of the groups of connected tentative active voxels, with spatially connected tentative active voxels belonging to the same group and a minimum size threshold (often 4). If a group of tentative active voxels is less than the threshold, all voxels in this group are removed, resulting in a final list of active voxels. Pseudocode is presented in Supplementary Table 1.

Our event detection can be roughly described as finding bumps in space–time, and it could be asked why we do not simply set a threshold on the ΔI data.
whether these two seeds should be merged and thus belong to the same event, we need to determine another seed. This scenario could happen when one event begins before the other super-voxel has a value that is considered close to the baseline before meeting a voxel with value that is considered close to the baseline before meeting the voxel with the largest $d^a$ peaks. For example, two super-voxels have the same intensity value. This is helpful for the case in which some pixels have saturated values. Because pure random fluctuation can also lead to local maxima, we restrict the search of local maxima to activate voxels only (see pseudocode in Supplementary Table 1). The resultant local maxima are considered peaks for the purpose of peak detection, the subsequent step in the algorithm. We start peak detection by identifying the local maxima (as discussed above) because local maxima are likely to contain the strongest signal and thus have a better SNR than other points. The 3D Gaussian smoothing is used to further improve SNR, motivated by the fact that an event occupies multiple pixels and spans multiple time points.

Step 4 (detecting peaks and their spatiotemporal extent). We partially and temporally extend each seed detected above to all voxels that are spatially associated with each event. We call the collection of the seed and its extended voxels the super-voxel. Seeds are processed one-by-one, with higher-intensity seeds processed first. Each seed is first extended temporally, then spatially. The spatiotemporal index $(x_i, y_i, t_i)$ denotes the seed. When we temporally extend the seed backwards and forwards (Supplementary Fig. 2b and Supplementary Table 2), we encounter two main scenarios. In the first, a voxel before $(x_i, y_i, t_i)$ has a value close to the baseline $F_0$ and a voxel after $(x_i, y_i, t_i)$ is also close to $F_0$. If a voxel has an intensity <20% of the seed value, it is defined as close to baseline. In this scenario, the seed is extended temporally until it reaches these two voxels. In the second scenario, extension occurs if at least one of two directions never meets a voxel with value $x$ that is considered close to the baseline before meeting another seed. This scenario could happen when one event begins before the previous event drops completely to the baseline fluorescence level. To determine whether these two seeds should be merged and thus belong to the same event, we use $F_{1 \& 2}$ to denote the minimum value between the two seeds and calculate the difference between $F_{1 \& 2}$ and value at the end $(x_i, y_i, t_i)$. If the difference is larger than the threshold $\Delta_x$ (where $t_1$, $t_2$ indicates time window), which is 2min by default for most data, the minimum is considered the end of the extension. Otherwise, these two seeds are merged and the extension continues. For very high peaks, this threshold is too low for perceptually meaningful separation. To split two adjacent high $d^a$ peaks, we require that the time difference allowed reaches 10.

Step 5 (clustering peaks to identify candidates for super-events). A super-event is defined as a group of events connected spatially but originating from different initiation locations. This step was motivated by the frequent observation in real data that multiple events can be spatially connected at some time point. One example is a large burst in the in vivo dataset, where multiple events start at different places but merge as a burst at a later stage. Another example is a set of two events originating from different places, propagating and meeting each other in the middle (Supplementary Fig. 3). Thus, in a spatial direction, we may encounter multiple events within the super-event. However, we never encounter two or more events in the temporal direction, which guides the following algorithm. To identify candidates for super-events, we first cluster peaks, but these results are not identical to super-events, because voxels extended to be associated with the peak may have some overlap. Therefore, we threshold any overlap below, the candidate super-event must be purified to resolve the final super-event. Since each super-voxel extends from its seed (representative peak), we also call the process of clustering super-voxels clustering peaks for conceptual convenience. If two super-voxels are connected and their rise-time difference is less than a given threshold, they are considered super-voxels because the two super-voxels, if 10% of pixels of either super-voxel are also occupied by the other, they are a conflicting pair. For each super-voxel, we list all of its neighbors and conflicting counterparts. To cluster peaks/super-voxels (Supplementary Fig. 2b and Supplementary Table 3), we begin with the earliest occurring super-voxel and check each of its neighbors. If a neighbor is not conflicting with that super-voxel, it is added to the super-voxel. If the added super-voxel can be added, then we move to the next earliest super-voxel that is not added to any others, and repeat this process. An iterative approach prioritizes events that are close to each other. Supposing the largest rise-time difference for super-voxels that is allowed for them to be neighbors is 10, we start the procedure with the allowed difference as 0 and merge the super-voxels. Then we increment the allowed difference by 1 and repeat the step above, until the rise-time difference allowed reaches 10.

Step 6 (estimating signal propagation patterns). For each spatial location/pixel, an associated time series indicates the signal dynamics. Estimation of propagation patterns is formulated as a mathematical problem of time alignment between the time series at each location and a representative/reference time series. Time alignment results directly delay the relay of a given pixel at a time frame with respect to the representative dynamics. Conventionally, time alignment is accomplished by dynamic time warping. However, dynamic time warping is notoriously time consuming, which leads to unreliable propagation estimation. Since two adjacent pixels have more similar propagation patterns than two distant pixels, we impose a smoothness constraint on neighboring pixels using our recently developed mathematical model—GTW—to explicitly incorporate this constraint. However, since we do not have a representative time series at the very beginning, one way to tackle this problem is to guess a reference time series from the data and align time series at each pixel to this reference. Then, we use alignment results to obtain an updated reference, and iterate the process of alignment and update of reference until it converges (Supplementary Fig. 2b and Supplementary Table 3).

To initialize the reference time series, we search for the voxel with the largest $d^F$ value and record that voxel’s location. The initial reference is then estimated as the average time series of the pixels in the $5 \times 5$ square around that location, with the square size a user-tunable parameter. The voxel with the largest $d^F$ value is used because it has the best SNR. We do not use the time series at a single pixel to initiate the reference because it is noisy, nor do we use the average time series over all the pixels, because the average would be a large distortion to the original signal. The reference is represented as $\Omega = (x_i, y_i, t_i)$, the average of the neighborhood graph and the reference time series to GTW to calculate the time alignment between all pixels and the reference. For each pixel, we consider the eight pixels around the $3 \times 3$ grid as neighbors. A GTW parameter controls the balance between fitness and smoothness of the alignment. We empirically found 1 to be a good value. To control computational complexity, GTW has another parameter corresponding to the size of the super-voxel. In our experiments, we found no time delay induced by propagation larger than 11 frames. So, we set that parameter to 11.

Step 7 (detecting super-events). Once the time alignment between the representative dynamics and the time series at each pixel is obtained, we refine candidate super-events to obtain final super-events. A super-event is defined as detected when the representative dynamics and all voxels are associated with the super-event. Since each voxel is jointly specified by spatial location and time frame, we next determine which pixels and time frames jointly belong to the super-event. Since representative dynamics are already obtained in the previous step,
of propagation estimation, here we focus on how to determine which pixels and which time frames are covered by the super-event.

Because each pixel corresponds to a time series, if a pixel belongs to a super-event, the time series should be highly correlated to the representative dynamics of the super-event. Note that the correlation is calculated based on the aligned time series to account for the time distortion due to signal propagation. Thus, we first obtain a new time series for each pixel based on the time alignment obtained previously. Then, we calculate the Pearson correlation between each new time series and the representative dynamics, leading to a correlation map. We further convert the Pearson correlation to z-score using Fisher’s transform.

Here, we do not use a threshold for each z-score to determine whether that pixel is statistically significantly associated with the super-event because that ignores the neighborhood information in the correlation map and is less statistically powerful. Instead, incorporating the information from the neighboring pixels, we apply our recently developed order-statistics-based region-growing method to determine which pixels should be associated with the super-event (Supplementary Fig. 2b and Supplementary Table 3).

To determine which time frames are associated with the super-event, we now examine the representative time series, calculating the maximum intensity along the curve and considering all time frames with intensity above a certain threshold (the value at time $t_1$, here $m$ is arbitrary). The distance between pixel $i$ and pixel $j$ induced by this path is therefore defined as $\max(t_1 - t_1, t_1 - t_1)$. If the distance induced by any path is less than the given threshold, these two local minima are merged.

We next separate super-events into individual events by simultaneously extending all remaining local minima. Each remaining local minimum corresponds to one event. Pixels attached to a local minimum are defined as growing. With each iteration, we add the earliest occurring pixel to a growing event. If the pixel under examination is adjacent to a growing event, it is added to that growing event, and then we find the next earliest occurring pixels. Otherwise, we add it to the walklist and continue with the next earliest occurring one. Each time a pixel is successfully added to a growing event, pixels in the walklist are checked as to whether they can be added to growing events. When the growing process ends, all individual events are obtained (see pseudocode in Supplementary Table 3).

Run-time analysis of AQuA algorithm. In our implementation, ~60–70% of the total running time is spent on propagation estimation, and super-event detection and splitting. The super-voxel detection step takes about 15% of the total time. Another 7% of the time extracts features from each detected event. The event cleaning and post-processing takes ~6% of the total time, while it takes <1% of the time to load the data and <2% for the active voxel step, which finds baseline, estimates noise and gets seeds. Tested on a desktop computer with Intel Xeon E5-2630 CPU and 128 GB memory using Windows and MATLAB version R2018b, the total running time ranges from several minutes to 1.5 h, depending on file size and data complexity (Supplementary Table 4).

Generation of simulation datasets. Spatial footprint templates. We built a set of templates for event footprints from real ex vivo data that serve as the basis for the ROI maps in the subsequent step. Footprints are processed by morphology closing, hole filling and morphology opening to clean boundaries, with 1,683 templates are obtained (see pseudocode in Supplementary Table 3).

Event generation. We simulate only one seed (starting propagation point) in each ROI. For each event, we generate a rise-time map (for each pixel in the ROI) and construct event-propagation based on the map. We obtain this map by simulating a growing process starting from the seed pixel, with the seed pixel active at the first time point. At the next time point, its neighboring pixels are active with a variable success probability. Growth continues until ≥90% of pixels in the ROI are included in the event. Based on the rise-time map, we identify frames at which pixels become active in the event. To determine when the event ends, we treat growing and moving propagation differently. In growing propagation, all pixels are inactive simultaneously two frames after the last pixel becomes active. For moving propagation, the duration is five frames. Typically, we generate approximately 140 events in 14 ROIs for each synthetic dataset.

Simulation dataset 1 (size-changing events). To simulate event size changes, we generate events for each ROI and then alter them to have different sizes so that each ROI in the 2D map will be related to multiple events whose centers are inside that ROI, but whose sizes are different. The degree of size change is characterized by the odds ratio (maximum = 5) between the maximum and the minimum allowable sizes of the events associated with that ROI. For example, with an odds ratio of 2, the size of the event will range from 50% to 200% of the ROI area. The chances for the event size to be larger or smaller than the area of the ROI are the same. To achieve this, we generate a random number between 1 and 2, then randomly assign whether to enlarge size by multiplying or shrink by dividing by this factor. Event duration is four frames.

To determine the frames at which the event occurs, we first put the event 10–30 frames (randomly) after the ROI occurs. Spatial distance of this event from others must be ≥3 pixels and temporal distance ≥4 frames. Part of the event may be inside the spatiotemporal footprint of other ROIs, as long as its spatiotemporal distance to other events is larger than the threshold set above. Events are generated for each ROI; on average, we simulate 250 frames with 800 events on 90 ROIs.

Simulation dataset 2 (location-changing events). To simulate event location changes, we generate events with the same size for each ROI and shift them to nearby locations. Thus, each ROI (450–550-pixel size) is related to multiple events near to that ROI. We use ‘dist’ to denote the distance between the event center and the ROI center. We use ‘diam’ to denote the diameter of the ROI. The degree of location change is quantified by the ratio between dist and diam. For example, if we set 0.5 as the maximum degree of location change, the distance of the center of a new event to the ROI will be 0.5–0.5 times the diameter of the ROI. If the ratio is 0, we simulate a pure ROI dataset. The new event may be located in any direction from the ROI, randomly picked from 0 to 2π. Shapes of new events are randomly picked from the templates, so may be different from the ROI, while size is constant. Event duration is four frames, and the remaining steps are the same as above. On average, we simulate 250 frames with 800 events on 90 ROIs.

Simulation dataset 3 (propagating events). We simulated two types of propagation: growing and moving, leading to three types of synthetic datasets: growing only, moving only and mixed. These three types are generated similarly. The ROI maps are generated as above and are removed by merging with spatially adjacent local minima. We use rise-time to determine whether two local minima should be merged. This idea can be illustrated with the following one-dimensional example: $[1, 2, 4, 2, 2]$. The two local minima are the first and the last pixels (pixels 1 and 5), respectively, occurring at times $t_1$ and $t_5$, respectively. To determine whether they should be merged, we find all paths connecting them. In this example, there is only one path and the pixel with the latest rise-time in this path is the third pixel (rise-time = 4; here $m$ is arbitrary). The distance between pixel 1 and pixel 5 induced by this path is therefore defined as $\max(t_1 - t_1, t_1 - t_5)$. If the distance induced by any path is less than the given threshold, these two local minima are merged.

We next separate super-events into individual events by simultaneously extending all remaining local minima. Each remaining local minimum corresponds to one event. Pixels attached to a local minimum are defined as growing. With each iteration, we add the earliest occurring pixel to a growing event. If the pixel under examination is adjacent to a growing event, it is added to that growing event, and then we find the next earliest occurring pixels. Otherwise, we add it to the walklist and continue with the next earliest occurring one. Each time a pixel is successfully added to a growing event, pixels in the walklist are checked as to whether they can be added to growing events. When the growing process ends, all individual events are obtained (see pseudocode in Supplementary Table 3).

When we change the degree of location change, size change and propagation duration, we add noise with 10 dB SNR. To study the impact of SNR on size changes, size-change degree is 3. For location changes, distance-change ratio is 0.5 while varying SNRs. For propagation, propagation duration is five frames. Seven SNRs are tested: 0, 2.5, 5, 7.5, 10, 15, 20 (all in dB).

Post-processing simulated data. We set the average signal intensity at 0.2, with a range 0–1. Synthetic data are spatially filtered to mimic blurred boundaries in real data. The smoothing is performed with a Gaussian filter with a standard deviation of $\sigma$ and signal intensity <0.05 after smoothing are removed. Remaining signals are temporally filtered with a kernel with a decay constant $\tau$ (the value at time $t$ is given by $\exp(-t \tau)$) of 0.6 frames. The rising kernel is linear. For propagation simulation, data are down-sampled by five. Next, we perform a cleaning step. For each pixel in each event, we find the highest intensity ($x_{peak}$) across frames. For that pixel, we set $x_{peak}$ to 0.2 times $x_{peak}$ to 0. Finally, a uniform background intensity of 0.2 is added (except for GECI-quant, where no background is added; see below under ‘Specific considerations for GECI-quant’).

Application of AQuA and peer methods on the simulation datasets. Based on our knowledge about simulated datasets, we apply specific considerations for each analytical method to set optimal parameters for each. In this way, we aim to assess the methodological limit of each method, rather than suboptimal performance due to inadequate parameter-setting. We expect that the performance of the peer methods on simulation data is an overestimate of their performance on real experimental data, because here we take advantage of the ground-truth knowledge, which is not available for experimental astrocyte data.
Event detection using peer methods. AQuA and CaSCaDe report detected events, while other methods report detected ROIs. For a consistent comparison, we detect events from those methods that use ROIs. Once ROIs are detected, we calculate an average score for each ROI; the curve is temporally smoothed with a time-window of 20. The minimum value in the smoothed curve is considered baseline. Assume the minimum value occurs at time \( t_{\text{min}} \). The baseline is then subtracted to obtain the \( dF \)/s noise. The noise standard deviation \( \sigma \) is estimated using 40 frames around \( t_{\text{min}} \). We then obtain a \( z \)-score curve as \( dF/\sigma \). A large \( z \)-score indicates an event; we use a \( z \)-score threshold of \( z_t \). The value \( z_t \) is set according to ground-truth knowledge, so that the smallest-size event in the simulation data is detected by this threshold. We use \( z_t \) and \( z_t \) to denote the peak intensity and the size for the smallest event in the ground truth. We also denote the ground truth noise level as \( \sigma_t \). Then, the threshold is calculated as

\[
\hat{z}_t = \min \left( \frac{0.99 \cdot z_t}{\sigma_t}, 10 \right)
\]

We clip the score to 10 to avoid setting large values for high SNR. For CaSCaDe, we supply this value as the peak intensity threshold parameter. Using the \( z \)-score curves and threshold, we detect events from ROIs for CaImAn, Suite2P and GECl-quant. For each \( z \)-score curve, we find all frames with values \( \geq \hat{z}_t \). Each frame is a seed for an event. We assume that the \( z \)-score for that frame is \( z_t \) and we search before and after that frame. If the intensity of the frame is \( > 2.02 \cdot z_t \), then we include that frame associated with the event. We continue with another seed frame to find another event. Note that if a frame is considered part of an event, we do not consider it as a seed for another event, even if \( > 2.02 \cdot z_t \). The spatial footprint is fixed for all frames in an event, based on the ROI detected. Combining spatial footprint and frames, we obtain events for each ROI and identify all voxels belonging to an event.

Parameter setting for AQuA. The parameters of AQuA are based on the ex vivo-GCaMP-cyto preset with the following modifications: for different noise levels, we set the spatial smoothing filter size in the 3D smoothing function (\( 5 \times 5 \times 5 \)) and overlap (\( 5 \times 5 \times 5 \)). Components to be found is set to \( 16 \). We set threshold \( \mu \) to denote the peak intensity for CaImAn and found the following set of parameters performed best on simulation data. As event size can be large, we enlarge the patch size, so patch size = \([128,128,128]\) and overlap = \([32,32,32]\). All other parameters are based on default settings. No spatial or temporal down-sampling is used. Adjusting these parameters did not impact results on our simulated data. We used v5.5/2018 downloaded from https://github.com/flatoninstitute/CaImAn-MATLAB.

Specific considerations for CaImAn. We experimented with different parameters for CaImAn and found the following set of parameters performed best on simulation data. As event size can be large, we enlarge the patch size, so patch size = \([128,128,128]\) and overlap = \([32,32,32]\). Components to be found is set to \( 16 \). Maximum size is \( 5,000 \) and the minimum size is \( 25 \). Decay time is \( 0.5 \). Other parameters are based on default settings. No spatial or temporal down-sampling is used. Adjusting these parameters did not impact results on our simulated data. We used v6/4/18 downloaded from https://github.com/cortex-lab/Suite2P.

Performance evaluation on the simulated data. To evaluate the accuracy of detected events, we quantify the IoU. We consider all event voxels, not only pixels as in ROI-based methods. For each detected event \( i \), we find all the ground-truth events that have common voxels with event \( i \). For each such ground-truth event, for example, event \( j \), we calculate an IoU score (also known as Jaccard index) between this pair of events as the following:

\[
\text{IoU}_{ij} = \frac{\text{Number}(\text{voxels in event } i \cap \text{ voxels in event } j)}{\text{Number}(\text{voxels in event } i \cup \text{ voxels in event } j)}
\]

When a detected event can be perfectly matched with a ground-truth event, its IoU score is 1. A score of 0 indicates that this pair of events has nothing in common. For each detected event \( i \), we find the maximum IoU score among all pairs between this event and a ground-truth event. We denote this maximum score as \( \text{IoU}_i \). Similarly, we can compute a score \( \text{IoU}_j \) for the ground truth event \( j \). The final \( \text{IoU} \) score is obtained by averaging over all events, including detected and ground-truth events. Supposing we have \( I \) detected events and \( J \) ground truth events, where \( I \) and \( J \) are not necessarily equal, we compute the final score as the following:

\[
\text{IoU} = \frac{\sum_{i=1}^{I} \text{IoU}_i + \sum_{j=1}^{J} \text{IoU}_j}{I + J}
\]

Each simulation is repeated ten times. The mean and 95% confidence interval (CI) of IoU score is calculated and plotted. The CI is calculated as \( \mu_{\text{IoU}} \pm 1.96 \cdot \sigma \), where \( \mu_{\text{IoU}} \) is the estimated mean and \( \sigma \) is the estimated standard deviation based on ten repetitive runs.

Open-source software for analyzing and visualizing dynamic fluorescent signals in astrocytes. Applying software engineering principles, we developed an open-source toolbox for astrocyte fluorescent imaging data with detailed user guidelines. The software not only implements the AQuA algorithm for detecting events, but also provides an integrated environment for users to see the results, interact with the analysis and combine other types of data such as cell/region masks and landmarks. There are two versions of the software with the same functionality, based on MATLAB or Fiji. The software is freely available at https://github.com/yu-lab-wfl/aqua where detailed documents and example applications can be found. A list of extracted features is shown in Supplementary Table 5. Highlights of the software follow.

First, the software implements AQuA and provides several options to export the event detection results, including TIFF files with color-coded events, event features in Excel, and MATLAB or Java data structures to be used by other programs. Second, the software can display analysis results by adding color to the raw video, where color encodes the value of a user-defined extracted feature such as propagation speed. Users can specify which feature to be displayed, either an existing feature in AQuA or a user-designed feature based on features provided by AQuA. We provide several predefined colormaps, but allow users to manually define colormaps as well. AQuA also provides a side-by-side view, to simultaneously display two features or a raw video plus one feature. Third, the software provides a convenient way to interactively view detected events and
their associated features. By clicking on an event, the dF/F curve for the event is shown in a separate panel below the video, and the time frames during which the event occurs are highlighted in red. The values of several other features for that event are also shown in another panel. The software allows multiple events to be selected simultaneously, so that their curves and features can be plotted together and compared. Fourth, the software provides both automatic and manual ways to proofread the results. For automatic proofreading, events are filtered by setting desired ranges for features-of-interest. Alternatively, users can choose the 'delete/restore' button and manually click an event to remove it. Fifth, the software provides flexible ways to incorporate cell morphology or landmark information. Users can manually supply cell morphology or regional information such as the cell boundary, which can assign events to individual cells. Users can also provide landmark information such as the location of a pipette for pharmacological application. Users can also load cell, region or landmark information from other data sources, such as another fluorescence channel that captures cell morphology. The software can extract landmark-related features for each event, including the direction of propagation relative to a landmark.

Statistics. The CIs in Fig. 2 and Supplementary Fig. 6 (CI, 95%) were estimated as two times the standard deviation based on ten independent simulations. The CIs in Supplementary Fig. 12 were based on five independent simulations. For imaging experiments, the following statistical tests were employed, as appropriate, and as indicated: one- and two-tailed t-test, Wilcoxon rank sum, chi-squared test, k-means clustering and the Freedman–Diaconis’s rule. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in previous publications4–8, and statistical significance was calculated using post hoc tests. Data distribution was assumed to be normal but this was not formally tested.

In terms of randomization, samples were allocated into experimental groups by cell-type expression of each individual fluorescent sensor, and ex vivo or in vivo methodology. Within each group, imaging datasets were collected at similar ages. Both male and female were used and randomly selected. Data collection and analysis were not performed blind to the conditions of the experiments. For GluSnFR studies, blinding was not possible, because cell-type expression is evident from the images themselves. Likewise, for GABASnFR and GRAB-NE experiments, blinding was not possible because we injected a single virus and the bath applied only the relevant neurotransmitter. For GCaMP imaging studies, blinding was not relevant because all data within each dataset were grouped and analyzed together. No animals or data points were excluded from the analyses.

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

Data availability
The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Code availability
The code has been released under GNU General Public License v.3.0 and is available at https://github.com/yu-lab-vt/AQuA.

References
37. Goldey, G. J. et al. Removable cranial windows for long-term imaging in awake mice. Nat. Protoc. 9, 2515–2538 (2014).
Reporting Summary

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Statistics

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

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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

- All imaging data were collected using PrairieView software, version 5.

Data analysis

- Data analysis was carried out using customize code (AQuA 1.0) based on MATLAB version 2018a, Fiji (version 1.52h), Caltracer (version 3 beta), GECI-quant (v1.0), CaScaDe, CalMAn, Suite2P, NoRMCorre, as indicated.

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

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The datasets generated during the current study are available upon request.

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| Sample size | No statistical methods were used to predetermine sample size for simulation or imaging experiments, but our sample sizes are similar to those reported in previous publications, and statistical significance was calculated using post-hoc tests. |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from analyses. |
| Replication | All numerical simulation studies were replicated at least ten times using different random seeds. Imaging experiments were replicated using multiple animals across multiple days. All attempts at replication were successful. |
| Randomization | Samples were allocated into experimental groups by cell-type expression of each individual fluorescent sensor, and ex vivo or in vivo methodology. Within each group, imaging datasets were collected at similar ages. Both male and female were used and randomly selected. |
| Blinding | For GluSnFR studies, blinding was not possible, because cell-type expression is evident from the images themselves. Likewise, for GABASnFR and GRAB-NE experiments, blinding was not possible because we injected a single virus and bath applied only the relevant neurotransmitter. For GCaMP imaging studies, blinding was not relevant because all data within each dataset were grouped and analyzed together. |

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| ☒ Animals and other organisms | |
| ☒ Human research participants | |
| ☒ Clinical data | |

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | CS7Bl/6 and Swiss Webster (mixed males and females) were used in this study as indicated. For ex vivo experiments, experiments were performed at ages P10–23. For in vivo experiments, experiments were performed at ages P50–100. |
|--------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Wild animals | The study did not involve wild animals. |
| Field-collected samples | The study did not involve samples collected from the field. |
| Ethics oversight | All experimental procedures were approved by the UCSF Institutional Animal Care and Use Committee. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.