

Supplementary Information

Companion to Selection and weighting change the interpretation of phagocyte uptake and optical imaging
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Scope and version history

V9 clarifies the pH-dependent uptake label, the reconciled denominator universe and the prospective microglial question in the main article. Table S4 is harmonized with the main text on shared optical vectors and average temporal similarity. Numerical results, all other table cells, equations and figures are unchanged. The final audit repeats the bounded numerical checks and includes a separate rank-based check of optical contrasts.

V8 reorders the main narrative around uptake participation and aggregate target load and adds a main figure displaying all eight count contexts under both inclusion rules. It preserves the numerical analyses and supplementary tables. Main Figures 1–3 of V7 are Figures 2–4 of V8. The new endpoint display is a presentation of existing results, selected after their analysis; full count trajectories remain in Figure S5. The new bounded verification checks extracted optical contrasts, reconciled count sums and recorded ordering rows without repeating source extraction or simulations.

V5 changed author metadata and journal presentation and added a replay of the existing conditional controls. V6 preserves those results, presents optical selections symmetrically and adds exploratory reconciliation of functional count sheets with an independent numerical audit. V7 preserves these numerical results and clarifies the fixed-offset permutation expectation and the distinction between time-origin invariance and signal–background mixing. New mathematical examples are explicitly illustrative, not experiments or fitted explanations. The V4 and V3 folders in the supporting materials retain the analysis history. References use the same numbering as the main Article.

This version retains the original descriptive methods and adds conditional controls developed after V3. The new sections distinguish the observed image associations from their conditional reference models; no original inference plan was rewritten.

This supplement preserves the full measurement definitions, all optical contexts, source comparisons and deterministic sensitivities behind the Article. References use the same numbering as the main Article. The original three analyses and their declared first-block extraction were retained. The second-block reconciliation and exact pooling decomposition were added after version 1 and are labelled subsequent exploratory audits. No new biological experiment, fitted-lifetime model or functional validation was introduced.

The first-block rule is an auditable analysis decision rather than a claim that this block is the publication's final population. Matching the second block to Prism and the publication makes both populations easier to identify; it does not explain their selection. Source inputs and the original numerical outputs remain byte-identical to the version-1 package.

Full methods

Design and resource selection

This was an exploratory secondary analysis of openly available data. Resource selection emphasized relevant phagocytes, accessible numerical inputs, documented acquisition information and tractable local execution. It was a targeted selection, not a systematic review or a representative sample of public microscopy. Module-specific plans were written before the new effect calculations, after knowledge of the source publications and prior resource scouting. They were not preregistrations. All planned contrasts and sensitivity settings are reported, including stable ordering and near-zero associations.

The biological module used five original files from the Bess Figshare project 163321, with numerical optical extraction from Co-Culture Data.xlsx and comparison against Co-Culture FLIM Results.pzfx [4,10]. The raw FLIM module used two macrophage control fields from Zenodo 14330511 [11,12]. A publicly linked Dryad resource associated with human dermal macrophage FLIM was considered but could not be retrieved through the tested public route; no measurements from it entered this analysis [7]. The confocal module selected all three component-3 records in BioImage Archive S-BIAD564 meeting the declared criteria: spinning-disk confocal acquisition, the lyn-tagRFPT reporter, a reported 60-second interval, a temporal TIFF of at least 30 frames, and a file size no greater than 125 MB [13,14]. Supplementary Table S1 identifies the resulting units and their limits.

Table S1 Resources and units

Resource and model	Observations	Comparison	Inference boundary
Bess Murine RAW264.7 co-cultures	First XLSX block: 182 entries per optical feature; Prism: 190; 3 features; 4 contexts	Active versus inactive within each activation and target context	No recoverable culture or field grouping; no future response
Musimbi Porcine PAM 3D4/31	2 raw fields; 227 arrival bins $\times$ 512 $\times$ 512 pixels each	5 intensity thresholds and photon versus pixel weighting	Uncorrected arrival moments; no cell segmentation or functional assay
Möller Zebrafish microglia	3 processed confocal records; 60, 82 and 120 frames	5 sampling intervals; all phases; 3 representations	Image-pattern sensitivity; no event rate or treatment inference

Optical state and uptake context

The source co-cultures used murine RAW264.7 macrophages, indexed by the authors' M0 medium-only and M1 interferon- γ -conditioned labels, and by CT26 or 4T1 tumour-cell targets [4]. We retain these experimental labels rather than interpreting them as universal macrophage classes. The deposited active and inactive (source label: non-active) labels refer to the source uptake assay. The primary paper describes a pHrodo-based acidic-compartment signal and masks used to exclude tumour-cell signal from the macrophage optical measurements. This operational label can depend on detectable internalized material and its environment; it does not measure future clearance capacity or complete degradation.

The three deposited features were optical redox ratio, mean NADH lifetime in nanoseconds, and the ratio of short- to long-lifetime amplitudes, A1/A2. The NADH feature name follows the deposit; the acquired NAD(P)H channel does not establish a chemically isolated NADH measurement [3,4]. Deposited redox values were retained without inversion. The precise numerator convention could not be verified in the retrieved methods or public code, so no direction of oxidation or glycolysis was assigned to that ratio.

Mean lifetime is the source fit-derived scalar, whereas A1 and A2 denote the relative short and long exponential contributions. We did not refit decays for this dataset. The separately analysed raw FLIM moments described below are different quantities.

The first explicitly labelled spreadsheet block, columns B, C, E and F in the six relevant sheets, was selected before calculating effects. This was a declared analytical choice, not a certified canonical cell population. A row required a source Cell label and a finite numeric value. Duplicate layout blocks were audited rather than concatenated. Summaries and unlabelled cells were excluded. Deposit-range checks required redox ratios between zero and one and positive lifetime or amplitude ratios. The redox range was not a verified universal physical domain because its exact definition remained unresolved. No finite labelled value, including unusual values affecting the result, was removed.

Within each of four contexts, active and inactive distributions were compared using medians, interquartile ranges and Cliff's delta. Delta is the fraction of all active–inactive pairs in which the active value is larger minus the fraction in which it is smaller; ties contribute zero. It ranges from -1 to $+1$. The corresponding empirical area under the receiver operating characteristic curve equals one half of delta plus one half, always with higher values assigned to the active class. Direction was not flipped to improve discrimination. These are descriptive pairwise orderings of deposited entries, not cross-validated classifier performance.

All 12 feature-by-context contrasts were retained. Pooled deltas and an equally weighted average of the four within-context deltas were computed to expose the effect of changing the comparison target. They are different estimands: pooling introduces comparisons across contexts and reflects the observed group mixture. Sensitivity checks used the alternative Prism tables, available secondary spreadsheet blocks, unique-value retention and deletion of one record at a time. A leave-one-record-out range measures influence on the observed statistic, not biological uncertainty. Culture, field and donor identifiers could not be recovered for the optical entries. We therefore calculated no biological p-values, confidence intervals or donor-generalization estimates. Trial and region identifiers in the separate uptake workbook were not transferred to the optical measurements.

Raw FLIM selection and aggregation

Two complete OME-TIFF files labelled as macrophage DMSO controls, fields i and ii, were retrieved from the Fig1_M0-vs-M1 archive directory [11,12]. The associated system was the porcine PAM 3D4/31 macrophage line. Only the first acquisition frame for each field was selected; these two fields do not establish independent biological replication. The source study combined five acquisition frames per field before its FLIM analysis. This first-frame audit does not reproduce that multi-frame processing [11]. Public HTTP byte ranges allowed extraction of complete ZIP members without downloading the full archive. Member CRC32 values and newly computed SHA-256 hashes were recorded. The whole-archive checksum was not verified because the whole archive was not retrieved.

Each physical TIFF contained 227 pages of 512×512 unsigned 16-bit counts. Custom metadata identified these pages as lifetime bins with a 0.054991 ns increment, although generic OME metadata named the dimension Z and referenced four additional files. We read only the pages physically present. External acquisition frames that were not retrieved were never filled with zeros or interpreted as observations. The header supplied a $200 \times 200 \mu\text{m}$ field size.

For each pixel, intensity was the sum of counts over all recorded bins. Masks retained positive pixels with intensity at or above within-field percentiles 0, 25, 50, 75 and 90; values tied at a threshold were retained. For each mask, the photon-weighted mean arrival was the sum of recorded arrival coordinates multiplied by their photon counts, divided by total retained photons. The equal-pixel mean instead gave each retained

pixel the same weight after calculating its own mean arrival. We report both quantities and the retained pixel and photon totals. No instrument response function, background or time-zero correction was applied. The resulting first moments are relative to the recorded instrument origin and must not be interpreted as fitted fluorescence lifetimes or biochemical rate constants.

Confocal retrospective temporal thinning

The three selected source records were processed maximum-intensity projections of zebrafish microglia from a study of neuronal efferocytosis [13,14]. They contained 60, 82 and 120 frames. A one-minute interval was corroborated by ImageJ metadata for km203; for km187 and km190 it was taken from the source inventory because the ImageJ interval was absent. One record carried the source PST-1 treatment label and two carried DMSO labels. Different treatments, fields and image dimensions precluded a biological treatment comparison. Original event annotations, where present, were preserved but were not used as validated events in this analysis.

Each frame was divided by its total intensity to describe the spatial distribution of image signal. The distance between two frames was half the sum of absolute pixel differences between their normalized images. This dimensionless quantity measures redistribution of the recorded pattern, not cellular speed or an efferocytosis rate. Summed successive distances divided by elapsed minutes defined image variation per minute. This image descriptor is not a biochemical or phagocytic activity rate.

Sampling intervals were 1, 2, 3, 5 and 10 minutes. Every possible retrospective starting offset, or phase, was evaluated for each interval. Each coarse sequence was compared with all native frames between the same first and last retained frames. The retained-variation ratio was the coarse accumulated distance divided by native accumulated distance over that shared window. By the triangle inequality, thinning cannot increase this accumulated distance when endpoints and representation are held fixed. Its reduction is therefore expected mathematically; the empirical questions are its magnitude, phase sensitivity and whether record ordering changes. Linear interpolation error at omitted frames provided a secondary descriptor. Sensitivity analyses used one fixed intensity normalization for the entire record and spatial Gaussian smoothing with a one-pixel standard deviation before frame normalization.

Reproducibility and interpretation

Original inputs were preserved. File receipts, module plans, source mappings, tidy tables, full sensitivity results and scripts are included in the accompanying local bundle. Photon totals were checked through separate aggregation paths. Rank calculations were checked independently, including a second extraction from native spreadsheet XML and rational arithmetic. Deterministic phase ranges and influence ranges are reported as such. No meta-analysis combined the three resources, and the two optical modules were not placed on a shared lifetime scale. Computational reproducibility, sensitivity analysis and biological validation are distinguished throughout; multiple pixels or cells without independent grouping do not supply biological replication [15].

Complete contextual contrasts

Supplementary Table S2 reports all 12 contrasts for the previously declared first spreadsheet block and its Prism alternative. Median differences and empirical AUCs for both selections are included to connect rank effects with source units. Full medians, quartiles, extrema and every sensitivity setting are retained in analysis/bess/effects_by_context.csv and sensitivity_effects.csv. The alternative population is described as publication-aligned, not as an independent replicate. Features are not paired across rows to train a multivariate model because persistent identities were not established.

Table S2 Complete optical contrasts for both deposited selections

Feature	Context	n X	n P	δ X	δ P	Median Δ X	Median Δ P	AUC X	AUC P
Redox	M0/CT26	19	30	+0.446	+0.382	+0.089	+0.043	0.723	0.691
Redox	M1/CT26	15	7	-0.024	-0.014	-0.012	-0.012	0.488	0.493
Redox	M0/4T1	6	6	-0.389	-0.389	-0.088	-0.088	0.306	0.306
Redox	M1/4T1	22	27	+0.579	+0.358	+0.078	+0.067	0.789	0.679
Lifetime (ns)	M0/CT26	19	30	+0.379	+0.378	+0.077	+0.101	0.689	0.689
Lifetime (ns)	M1/CT26	15	7	+0.249	+0.314	+0.098	+0.106	0.624	0.657
Lifetime (ns)	M0/4T1	6	6	-0.289	-0.289	-0.190	-0.190	0.356	0.356
Lifetime (ns)	M1/4T1	22	27	+0.545	+0.627	+0.269	+0.274	0.773	0.814
A1/A2	M0/CT26	19	30	-0.393	-0.344	-0.641	-0.511	0.304	0.328
A1/A2	M1/CT26	15	7	+0.311	+0.257	+0.218	+0.218	0.656	0.629
A1/A2	M0/4T1	6	6	+0.267	+0.267	+0.252	+0.252	0.633	0.633
A1/A2	M1/4T1	22	27	-0.515	-0.583	-0.926	-1.057	0.242	0.209

X = first XLSX block; P = Prism export. n gives active entries; each inactive group has n = 30. Delta and AUC are dimensionless. Median change is active minus inactive in source units; fitted lifetime uses ns. Both selections are displayed symmetrically and are not independent replicates.

Subsequent reconciliation of source populations

All nine active-group vectors that differed from the first block matched the second block in order. For each of the active redox, fitted-lifetime and A1/A2 sheets, H2:H31 matched Prism M0/CT26, I2:I8 matched M1/CT26 and L2:L28 matched M1/4T1. Relative to the first block, these groups contained eleven and five additional entries per feature for M0/CT26 and M1/4T1, and eight fewer for M1/CT26. These are differences in scalar entries, not independently established counts of physical cells. The M0/4T1 active groups already agreed with the first block.

All 12 inactive vectors agreed between the first block and Prism. Separately, every one of the 12 explicitly reported optical mean–dispersion pairs in the source Results paragraph was matched by Prism means and sample standard deviations at three decimal places; the first block matched four. The complete set of comparisons is recorded in reviews/provenance/published_summary_comparison.json. This agreement supports the term publication-aligned alternative without certifying cell selection, identity or grouping.

Public Figshare metadata contained only version 1 for the Excel, Prism and code items. No accessible version narrative, workbook comments or experiment identifiers resolved the population differences. Redox.pdf calls calculateRedoxRatio but does not supply its definition. The exact numerator, normalization and averaging convention remain unresolved. The code PDFs were read as documents; they were not executed. A response from an attempted article supplement URL was HTML and was not treated as a successfully retrieved DOCX.

The original five data hashes remained intact. A second agent independently read native XLSX ZIP/XML and Prism XML, confirming all nine vector correspondences in order at absolute tolerance 10^{-12} . This is an independent computational extraction, not human peer review or biological validation. Scripts and complete evidence reside in reviews/provenance/ and reviews/verify_export_blocks.py.

Exact decomposition of the pooled rank contrast

Pooling changes both which contexts are compared and their weights. For each feature, 62 active and 120 inactive entries generate 7,440 pairs: 1,860 within a context and 5,580 across contexts. The within-context component weights each contextual delta by its active count multiplied by its inactive count. Because every context has 30 inactive entries, those weights are proportional to active counts here. They are not equal across contexts.

The pooled delta equals 0.25 times the pair-weighted within-context delta plus 0.75 times the cross-context delta. This identity follows by partitioning the complete pair set. In contrast, the equally weighted contextual mean gives each of the four contexts weight 0.25. The two changes must be distinguished when comparing pooled and equally weighted effects.

Table S3 Exact decomposition of pooled contrasts (first XLSX block)

Feature	Equal-context δ	Pair-weighted within δ	Cross-context δ	Pooled δ
Redox	+0.152767	+0.298387	+0.286201	+0.289247
Lifetime (ns)	+0.221100	+0.341935	+0.228495	+0.256855
A1/A2	-0.082589	-0.202151	-0.188530	-0.191935

For redox, changing from equal contextual weights to pair weights changes delta by +0.145620; adding the cross-context component changes it by -0.009140. The net pooled-minus-equal difference is +0.136480. These are algebraic descriptions of the observed pairs, not causal attributions. The calculation explicitly traversed all 22,320 pairs across three features using rational arithmetic and checked the original results. The exact fractions and all comparisons are available in reviews/pooling_decomposition.json and .csv. This audit was performed after version 1 and was not retrospectively labelled as planned.

What each resource establishes

Table S4 Interpretation across the three resources

Resource	Mathematical property	Empirical evidence	Functional ceiling
Bess co-cultures	Pooled pair contrast partitions exactly into within and cross components	Contextual sign differences; nine contrasts retain their signs after the active vector changes; three comparisons are identical by construction	Unknown grouping and selection; uptake label is not later clearance
Raw macrophage FLIM	Weighting difference equals intensity-arrival covariance divided by mean count	Mask shifts depart from fixed-count arrival exchangeability	Association may include background and instrument effects; no metabolic attribution
Microglial confocal	Thinning cannot increase accumulated distance; kR is a ratio of mean edge lengths	Original temporal neighbours are more similar on average than arbitrary pairs; ordering survives the evaluated temporal permutations	Image organization and hierarchy do not establish event or treatment effects

The common contribution is a set of worked audits of interpretation. The resources provide no paired multimodal cohort, pooled biological estimate, optimal acquisition interval or validated future-clearance predictor. Context-specific and multivariate predictors remain possible but were not tested.

Reproducibility and file map

Supplementary Data 1 includes retrieval receipts, hashes, module plans, code and complete CSV/JSON outputs. The selected raw images remain in their original public repositories and the separate full local bundle. For raw FLIM, member CRC32 checks verified the two recovered ZIP entries; the checksum of the full archive was not verified because the full archive was not downloaded. The custom TIFF header supplies the arrival-bin semantics. For confocal, the source inventory supplies intervals where ImageJ metadata omit them. These evidence dependencies remain explicit.

The version-1 replay reran all three modules in independent copies: 19 Bess numerical files, seven raw-FLIM numerical files including two NPZ archives, and six confocal numerical files matched at the declared tolerance. Twelve figure files were regenerated, not twelve distinct biological figures. Original inputs and numerical outputs were preserved. This tested the recorded local environments, not a clean installation. Version 2 preserved those numerical outputs and independently verified its additional source and pooling audits. Reproducibility commands and pinned dependencies are in reproducibility/README.md; the version-2 verification receipt is in reproducibility/v2_verification.json.

Conditional controls added after version 3

The following two controls were motivated by results already seen in V3. Written module plans preceded the new calculations. Seeds and simulation sizes were fixed in those plans. The analyses are exploratory and conditional on the selected finite fields and recordings; no new biological replicate was introduced. Versions 1–3, their plans, source files and original results were preserved. These controls do not constitute retrospective preregistration or an independent validation set.

Arrival reassignment with fixed pixel counts

Let I_p be the recorded count in positive pixel p , P the number of such pixels and $N = \sum_p I_p$ the total field count. Let m_p be its mean recorded arrival coordinate in ns. The photon-weighted field mean is $\mu_{\text{photon}} = \sum_p I_p m_p / N$; the equal-pixel mean is $\mu_{\text{pixel}} = \sum_p m_p / P$. Covariance and expectation in the next identity weight the P positive pixels equally and use the population denominator P . Then $\mu_{\text{photon}} - \mu_{\text{pixel}} = \text{Cov}(I, m) / E(I)$. This is an algebraic identity, not an additional independent observation. Its numerical discrepancy was below 4×10^{-16} ns in both fields.

The conditional reference fixes I_p and the multiset of N arrival coordinates recorded anywhere in the field. Uniform reassignment of that multiset among photon positions removes association with pixel identity while retaining the observed brightness map, all count-defined masks, and the global arrival histogram. It is deliberately not a complete detector or emission model. Background–signal mixtures, spatial instrument variation and biological heterogeneity can each violate this reference. Results therefore address the exchangeability assumption, not the absence of all noise.

For a fixed mask containing M photon positions, its arrival histogram under reassignment has a multivariate hypergeometric distribution with category counts equal to the global bin histogram and draw size M . NumPy 2.3.5 Generator.multivariate_hypergeometric, method marginals, generated 1999 independent realizations per nontrivial mask, seed 24092026. The five mask percentiles were 0, 25, 50, 75 and 90; threshold ties were included. Mask simulations were generated separately, not as jointly nested samples. Reported 2.5th and 97.5th percentiles are pointwise conditional references, not simultaneous bands. The complete mask produces an identically zero contrast and is represented exactly as zero in the new outputs.

Let $S^2 = \sum_j (t_j - \mu_{\text{photon}})^2 / (N-1)$, the finite-population sample variance of the N recorded coordinates. For a fixed mask, $\text{Var}(\mu_{\text{mask}} - \mu_{\text{photon}}) = S^2(1/M - 1/N)$. For equal-pixel versus photon weighting, $\text{Var}(\mu_{\text{pixel}} - \mu_{\text{photon}}) = S^2[(\sum_p 1/I_p)/P^2 - 1/N]$. Both expressions include finite-population dependence. They follow from $\text{Var}(\sum_j a_j t_{\text{perm}(j)}) = S^2[\sum_j a_j^2 - (\sum_j a_j)^2/N]$ for fixed position coefficients. Zero-count pixels are excluded from pixel means.

The formulas were checked independently using 50-digit Decimal arithmetic applied to original decay CSVs and a separate coefficient expansion. A four-position example with arrival coordinates 0, 1, 4 and 7 and pixel counts 1 and 3 was completely enumerated over 24 permutations using rational arithmetic; both variances equalled 5/6. This is a mathematical check, not synthetic experimental evidence. The conditional standard deviations for equal weighting were 0.001617 and 0.002248 ns; observed differences were 0.105347 and 0.126843 ns. Such ratios measure departure on this conditional scale, not biological effect sizes or discovery significance.

Table S5 Conditional arrival reference for Q90 masks

Field	Photons in Q90	Shift ns	Conditional SD ns	Reference 2.5–97.5% ns	Absolute exceedances
1	793,813	-0.134976	0.002427	-0.005007 to +0.004896	0/1999
2	537,297	-0.099287	0.002985	-0.005753 to +0.005872	0/1999

All nontrivial masks had zero absolute exceedances in 1999 realizations. This count does not mean $p = 0$ or establish a population test. The selected Q90 shifts were -0.134976 and -0.099287 ns; their direction reflects earlier recorded coordinates in bright pixels. No fitted lifetime, cofactor separation, cell segmentation, instrument-response correction, background correction or time-zero calibration was introduced. The source five-acquisition-frame processing was not reproduced by using two first frames.

Common time shifts and signal and background mixtures

Consider the same recorded photons and fixed intensity-based mask. Adding the same constant c , in nanoseconds, to every arrival coordinate increases both the photon-weighted mask mean and the whole-field mean by c . Their difference is unchanged:

$$(\mu_{\text{mask}} + c) - (\mu_{\text{field}} + c) = \mu_{\text{mask}} - \mu_{\text{field}}$$

Here μ_{mask} and μ_{field} denote means over photons in the mask and the full field. The same cancellation holds for the reassignment-reference differences when the same observations, masks and weights are used. This is an arithmetic relabelling of existing coordinates. It does not assert invariance to spatially varying offsets, a changed acquisition window, truncation, detector response or corrections that change the observed photons. Estimating fitted fluorescence lifetimes instead requires an explicit decay model and justified treatment of timing and instrument response.

A different issue is compositional mixing. In an illustrative pixel p , let S_p and B_p be nonnegative counts of signal and background photons, with positive total I_p . Let μ_s and μ_b be their respective mean arrival coordinates in nanoseconds. The mean of the combined recorded photons is:

$$m_p = \frac{S_p \mu_s + B_p \mu_b}{S_p + B_p}, \quad I_p = S_p + B_p$$

Even if μ_s and the signal distribution are common to all pixels, a changing signal fraction $S_p/(S_p+B_p)$ can relate brightness I_p to mean arrival when μ_s and μ_b differ. For example, holding background fixed and increasing signal changes both quantities. Not every change in mixture produces an association; when

the component means coincide the mean is constant. This is a conceptual counterexample to identifying metabolic heterogeneity from the association alone. Neither component nor its count was estimated from the two fields, and the example does not establish that background explains the observed shifts.

Temporal reassignment with fixed complete images

The confocal reference preserves each entire recorded image and its per-frame total-intensity normalization. Pairwise distances were calculated at full spatial resolution as half the L1 distance between normalized frames. The three symmetric distance matrices contain 3321, 7140 and 1770 unordered pairs for km187, km190 and km203. Permutation changes temporal positions, preserving the image collection and every pairwise distance. It destroys temporal association from biological dynamics and from acquisition trends alike.

For interval multiplier k and starting offset, define R_k as the accumulated retained-edge distance divided by the sum of all native-edge distances between the same endpoints. The retained window contains k times as many native edges as retained edges. Therefore $kR_k = \text{mean retained-edge distance} / \text{mean native-edge distance}$. The triangle inequality ensures $R_k \leq 1$. Neither property is an empirical biological finding. Exchangeable positions give the same marginal pair-distance distribution at different lags. For a fixed matched window, $1/k$ is the ratio of the expected coarse and native accumulated distances; it is a reference scale, not the expected retention.

Using seed 20260924 and PCG64, 499 independent frame permutations were generated per record, processing records in alphabetical order. Every iteration evaluated intervals of 1, 2, 3, 5 and 10 minutes and all starting offsets, always matching native and thinned endpoints. Medians over offsets were calculated within an iteration before summarizing the 499 iterations or ordering records. The permutation bands show pointwise 2.5th–97.5th percentiles. Iterations, offsets and intervals are not biological replicates. No population p -values or between-animal confidence intervals were calculated.

Table S6 Ten minute temporal retention and its reference

Record	Original median R	Permuted median R	Reference 2.5–97.5%	Permutations
km187	0.196985	0.099813	0.092113 to 0.107815	499
km190	0.222230	0.100369	0.092710 to 0.109140	499
km203	0.191064	0.100951	0.086549 to 0.112986	499

Across all 2495 interval-by-iteration ordering rows, the hierarchy by image variation per minute was $\text{km190} > \text{km187} > \text{km203}$. In each of 499 iterations, it also persisted jointly across all five intervals. This statement concerns offset medians of the finite evaluated references; it does not assert invariance to every possible permutation or every individual offset combination. The original V3 all-offset sensitivity result and this new median-based permutation result are distinct analyses.

The original adjacent-distance/all-pair-distance ratios were 0.405341, 0.301683 and 0.442424. Original R_k medians exceeded the pointwise 97.5th conditional reference percentile for all twelve record-by-nonnative-interval comparisons. These are correlated descriptive comparisons within three selected records. They show temporal organization of the recorded images, without assigning its source to motion, engulfment, drift, bleaching, background or structured noise. Retained image variation is neither an event-detection fraction nor a fraction of biological information preserved.

Twelve implementation checks passed, including source preservation, matched durations, R bounds, the kR identity and agreement with the original primary-representation metrics at the prespecified tolerance of 10^{-7} . Maximum discrepancy from V3 was below 1.18×10^{-9} ; the ratio-identity error was below 4.5×10^{-16} . An independent computational review recalculated adjacent and all-pair summaries and ten-minute retention from the stored matrices, and counted all 2495 ordering rows. That review did not independently reconstruct every pixel distance or rerun all permutations.

Expected variation at a fixed sampling offset

Let n be the number of stored frames and let the mean distance over their $n(n-1)/2$ unordered distinct index pairs be $\bar{d}_{all}$. Distances are dimensionless because they compare per-frame normalized intensity distributions. A uniform permutation assigns a uniform pair of distinct frame indices to any two different positions. Consequently, each retained edge has expected distance $\bar{d}_{all}$, whether or not the edges are mutually independent.

Fix an interval multiplier k and a starting offset r with q retained edges, where q is at least one. Let Δt be the native interval in minutes. For that fixed offset, $A_{(k,r)}$ is the sum of retained-edge distances divided by the fixed elapsed duration $q k \Delta t$. Linearity of expectation then gives:

$$E[A_{k,r}] = \frac{\bar{d}_{all}}{k \Delta t}$$

Here E denotes expectation over uniform permutations of the finite set of frame indices, distinct from the target-count symbol in the assay section. The mean distance $\bar{d}_{all}$ was 0.07023201255321743, 0.1428801626022682 and 0.045874884131706616 for km187, km190 and km203, respectively. The existing distance matrices reproduce these values. At a common interval their expected variations therefore order km190 > km187 > km203; at the reported one-minute native cadence, the expected value is $\bar{d}_{all}/k$ per minute. This explains how differences in overall image dispersion can sustain an ordering without the original temporal sequence. It does not prove that each realization or the medians across offsets must have that ordering.

The equation concerns the expected arithmetic variation for one fixed offset. It is not an identity for the expected median across offsets, and it is not an identity for the expected retention R , whose native-distance denominator depends on the permutation. In particular, it does not establish $E[R]=1/k$. The original and permuted offset medians in Figure 4 and Table S6 remain the observed finite-reference summaries. An exhaustive rational example included with the clarification code checks the fixed-offset equation and supplies counterexamples to substituting it for a median or ratio expectation. The example is mathematical, not an additional microscopy observation.

Data coverage and interpretation opportunities

The earlier dataset audit identified count-sheet inconsistencies that motivated the V6 analysis below. That audit remains in V4/reviews/dataset_information_audit.md; its original observations and limitations have not been overwritten.

The two selected raw FLIM series enumerate ten acquisition files; two first files were analysed. Eight remaining files would permit technical repeated-acquisition checks after their time coordinates are verified, not eight additional biological replicates. The archive Fig1 directory lists 100 TIFF files in twenty series prefixes, which do not establish twenty independent cultures.

The confocal component-3 inventory contains 67 entries, including 25 TIFF files. Three selected TIFFs contribute 262 frames. In the retrieved event annotations, all 24 km187 marks are spatially compatible with

its image dimensions, whereas only 12 of 27 km¹⁹⁰ marks fit directly within its crop. Neither coordinate compatibility nor a matching filename proves event alignment. Registration, time semantics and event meaning require verification before functional benchmarking. No annotation was used as ground truth in the current analyses.

These denominators describe files, frames or explicit table slots. There is no measured denominator for a percentage of biological information extracted. The three resources share neither cell identities nor a calibrated functional endpoint; removing one leaves the other numerical results unchanged. Their complementarity is methodological and does not demonstrate biological synergy.

Reproduction of the version 4 controls

The V4 package retains a read-only V3 baseline alongside the new control directories. `analysis/raw_null` contains the plan, executable analysis, plotting code, complete mask table and results JSON. `analysis/confocal_null` contains the plan, executable analysis, full pairwise matrices, all permutation and phase summaries, verification receipts and figure files. Original source arrays and their hashes remain in the V3 baseline. The distribution README distinguishes the compact code supplement from the full local review bundle with selected raw microscopy inputs. No clean-environment or human validation is asserted.

Functional count reconstruction added in version 6

The observation unit was a source target label $\times$ condition label $\times$ region-of-interest (ROI) label $\times$ nominal time. The two targets were CT26 and 4T1, the four source condition labels were M0, IFN- γ , LPS and M2, and each combination contained six ROI labels at nine times from 0 to 120 minutes in 15-minute increments. The manuscript uses IFN- γ for display while the extraction preserves IFN- γ exactly. Source headings use the same generic macrophage count labels under different conditions; these labels are not used to reclassify every cell as M1. The published study defines participation and target index [4]; the deposited worksheets also define E/P under the label capacity [10]. The audit does not claim these ratios or their identity as new estimators. Time zero is the first recorded image after co-incubation, not a pre-contact baseline. The source article is Bess et al., available at <https://pmc.ncbi.nlm.nih.gov/articles/PMC10959688/>; the source numerical files are in Figshare project 163321, https://figshare.com/projects/Live-cell_NADH_autofluorescence_during_phagocytosis/163321.

The percentage, index and capacity sheets contain copied raw-count grids. They were not concatenated: 1,296 copied records correspond to 432 nominal observational slots. The percentage sheet had 408 complete triples, the index sheet 406 and the capacity sheet 403. Five 4T1 slots at time zero differed in presence or absence of counts between sheets; all alternatives are retained in `'crosssheet_discrepancies.csv'`. Twenty-four slots were incomplete in the percentage grid. Of the remaining common records, 27 had N=0 and three had E<P, giving 373 eligible records. The E<P triples occurred in CT26/IFN- γ ROI 6 at 30, 45 and 60 minutes, with (N,P,E)=(39,29,14), (36,27,13) and (37,24,9). Their definitions or recording require clarification for the chosen distinct-target interpretation; their values were not changed. P=E=0 with N>0 was retained for participation and index, while the individual-record E/P was left undefined. Undefined ratios were never replaced by zero.

Restricting every context to labels eligible at all nine times retained 38 ROI sequences: CT26 M0, IFN- γ , LPS and M2 respectively contributed 5, 5, 5 and 6; the corresponding 4T1 counts were 2, 6, 3 and 6. This restriction fixes the set of ROI labels, not the cells present or the number of macrophages. Both this conservative summary and the available-ROI summary are supplied for every condition and time, with exact N, P and E totals and included ROI labels. Pooled participation weights by the number of counted macrophages, while pooled E/P weights by the number of participating macrophages. These differ from

unweighted means of ROI-specific ratios. No summation across time or target was used to define an experimental sample size.

A source-coordinate audit identified a distinct temporal reference mismatch in 54 summary-count formulas: nine formulas in each of six sheets, spanning N/P/E and M0/IFN-y/M2 at the row labelled 45 minutes, refer to raw counts labelled 30 minutes. For example, `CT26 - % Phagocytosis!A58` labels 45 minutes, whereas `B61` contains `=Z4+AF4+Z11+AF11+Z18+AF18`; these six cells belong to the raw-count block headed by `Y1`, labelled 30 minutes. The formulas are cached and summed consistently, but their referenced time differs from the summary label. The reconstruction used raw-grid coordinates and time labels, so it does not inherit those summary references. This observation describes the deposited workbook only. It does not establish that the publication or Prism trajectories inherited the same mismatch.

The Prism assay export was separately extracted into 486 numerical values with exact table identifiers, condition labels and anonymous subcolumn positions. For instance, the first M0/CT26 participation subcolumn contains 38.235% at time zero, whereas the reconstructed raw-grid total is 59.091%. This unresolved population correspondence prevents treating them as alternative replicates of an identified culture or selecting a Prism column to replace a worksheet value. Nine trial-labelled worksheets are templates: each has 324 empty ROI-count cells and 54 cached zeros in totals. Their Trial 1–3 labels therefore do not assign biological replicate identities to the populated metric grids.

The subsequent sensitivity retaining the three E<P triples yielded 376 eligible arithmetic records and 39 complete-time ROI sequences. Only CT26/IFN-y summaries changed; every M0/CT26 value, including the preselected worked example, was unchanged. These extra ratios are supplied separately and do not replace the primary load interpretation or figure. This sensitivity was proposed after inspection of the primary results and is explicitly identified as such.

Computational checks compared 3,888 source count-cell readings with a separate ZIP/XML reader, including blank cells, and compared 1,293 local metric formulas and 486 summary-count formulas. Exact Fraction arithmetic verified every defined participation $\times$ load identity at record and pooled levels. Adversarial classifier checks retained legitimate zero participation and rejected missing denominators, N=0, E<P under the primary criterion, inconsistent sheets and positive E with P=0. Original XLSX/PZFX and download-receipt hashes were unchanged. A separate agent independently checked the reconstruction; its report is distinct from this execution receipt and is not human peer review or experimental validation.

Composition sensitivity in M0 with 4T1 targets

At time zero, the complete first-sheet grid has N=60, P=7 and E=24, giving participation 11.7%. The unanimous available-ROI subset has N=29, P=7 and E=24 (24.1%); the two complete-time ROI labels have N=15, P=7 and E=24 (46.7%). The two first-sheet triples absent from other copied sheets have (N,P,E)=(23,0,0) and (8,0,0). These are availability differences, not contradictory numeric values. At 120 minutes both analytical subsets have N=16, P=6 and E=23 (37.5%). The available-ROI comparison rises, whereas the complete-time comparison falls. Neither subset eliminates selection, identifies the same cells or resolves the missing biological hierarchy. Both are reported without choosing one direction as the true response.

Limits of propagating the spreadsheet finding

In the source CT26/M2 assay at 45 minutes, Prism Table0 subcolumn 1 reports 11.429%, which agrees after rounding with the raw 45-minute grid (8/70), rather than 8.696% from the summary formula referring to 30 minutes. This is a concrete counterexample to assuming that the workbook reference discrepancy

propagates uniformly to Prism. It does not identify a replicate or establish the processing route of the published figures. Originals and cached values remain unmodified.

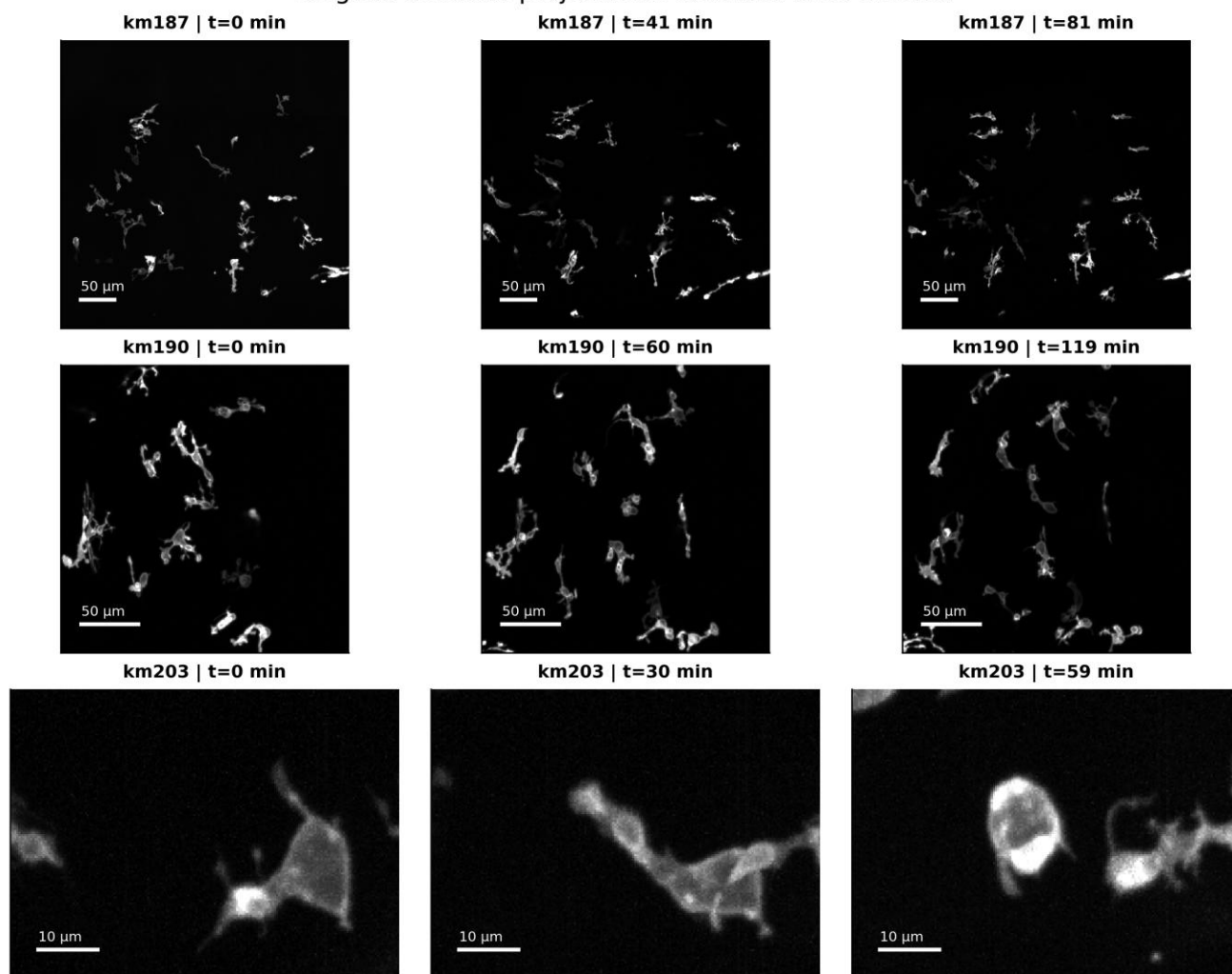
Table S7 Endpoint counts in the complete-time ROI sensitivity

Context	ROI count	N at 0 / 120 min	P at 0 / 120 min	E at 0 / 120 min	P/N (%) at 0 / 120 min	E/P at 0 / 120 min
M0/CT26	5	22 / 26	13 / 13	57 / 34	59.1 / 50.0	4.38 / 2.62
IFN- γ /CT26	5	321 / 378	170 / 109	818 / 281	53.0 / 28.8	4.81 / 2.58
LPS/CT26	5	156 / 148	32 / 10	106 / 48	20.5 / 6.8	3.31 / 4.80
M2/CT26	6	67 / 73	6 / 7	12 / 12	9.0 / 9.6	2.00 / 1.71
M0/4T1	2	15 / 16	7 / 6	24 / 23	46.7 / 37.5	3.43 / 3.83
IFN- γ /4T1	6	63 / 78	6 / 7	13 / 14	9.5 / 9.0	2.17 / 2.00
LPS/4T1	3	35 / 33	4 / 1	10 / 1	11.4 / 3.0	2.50 / 1.00
M2/4T1	6	164 / 197	6 / 9	16 / 19	3.7 / 4.6	2.67 / 2.11

Counts use the ROI labels eligible at all nine times under the primary rule. N = visible macrophages; P = source-labelled participants; E = labelled engulfed targets. Values separated by a slash refer to the first and 120-minute acquisitions. ROI counts do not denote independent cultures. Undefined E/P is retained when P = 0. All times, available-ROI summaries and subsequent E<P sensitivity are in the accompanying CSVs.

Figure S1 Original confocal projections and field context

Original confocal projections: field and time context

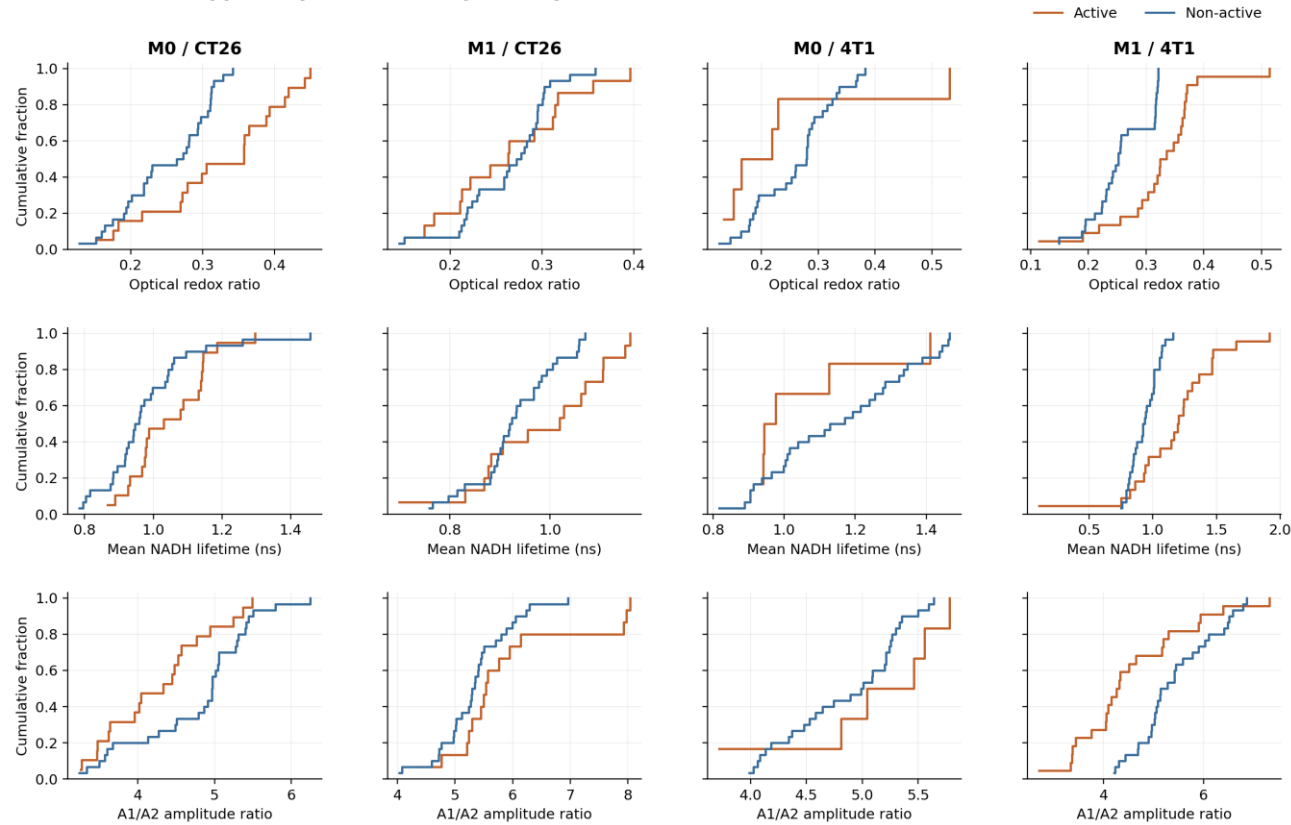


Display only: one fixed 1st-99.8th percentile window per complete record; numerical analysis uses stored intensities. Time is relative to first stored frame. Fields differ in size, composition and condition.

First, middle and last stored projections of each selected zebrafish record. Time is relative to the first frame, not treatment onset. A fixed 1st-99.8th percentile display window was applied per record; analysis uses original stored intensities. Scale bars are 50 μm for km187/km190 and 10 μm for km203. Source fields differ in size, condition and composition. Source: Möller et al. [13,14], CC0; new panel assembly.

Figure S2 All first-block optical distributions

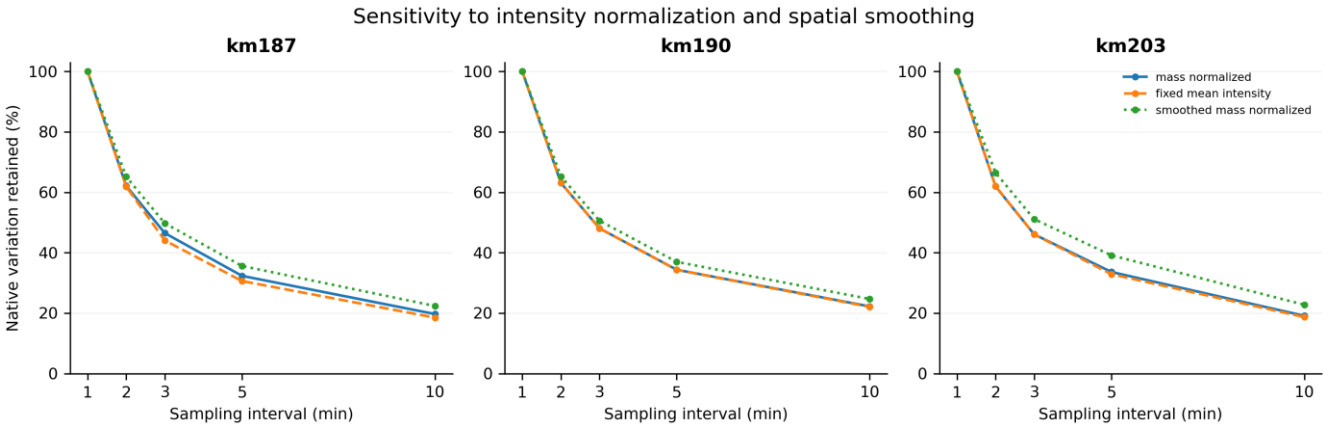
Distributions of supplied optical values by activity and context



Empirical cumulative distributions; no interpolation of missing records. Each panel is a separate context. Activity labels are inherited from the published pHrodo-SE mask procedure; no raw-image validation was possible.

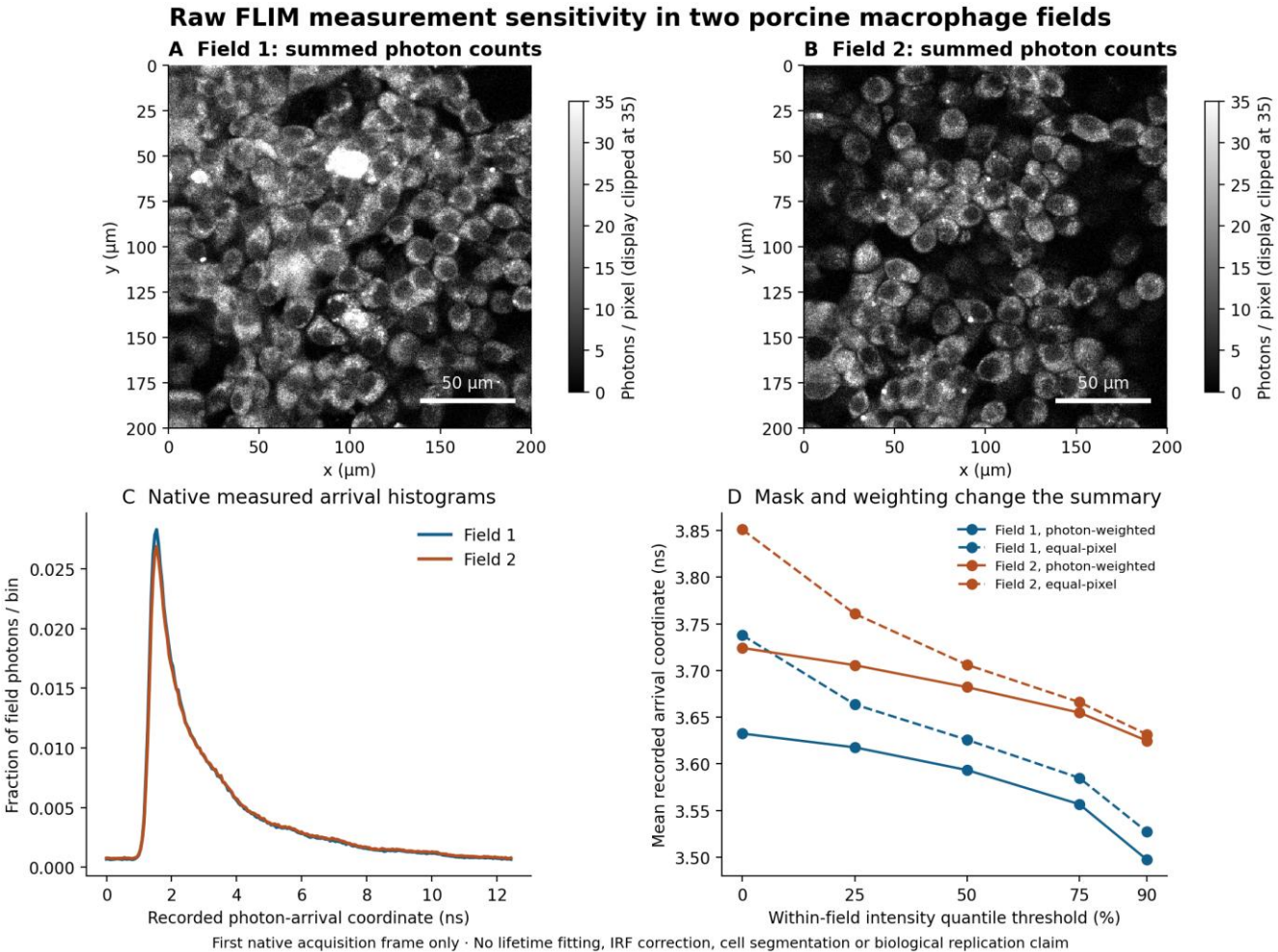
Empirical cumulative distributions for the first spreadsheet block. Each panel is one context and feature; values were not paired across features or interpolated to fill missing records. The active label is inherited from the source pHrodo procedure. Source: Bess et al. [4,10], CC BY 4.0; new descriptive analysis. The inherited plot label “Non-active” denotes the inactive category used in the main Article.

Figure S3 Alternative representations of confocal variation



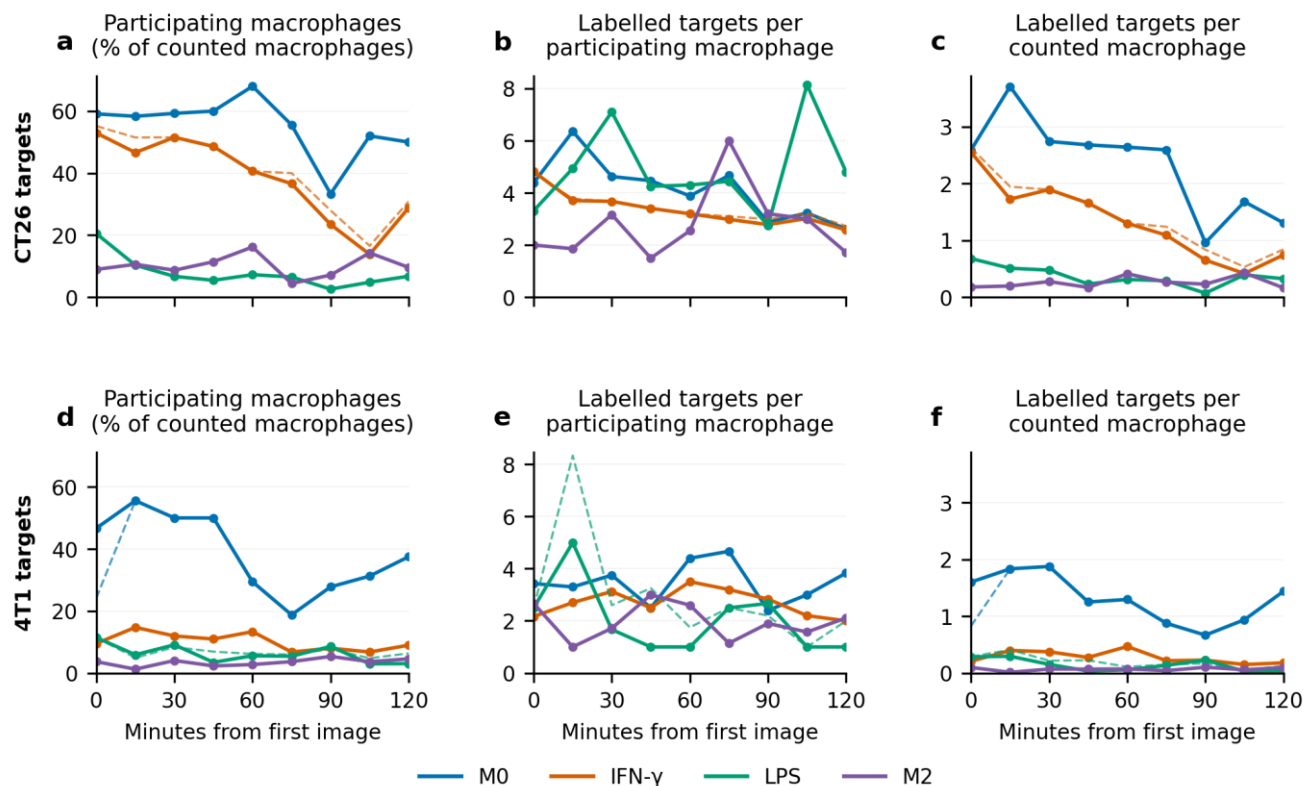
Phase-median retention under per-frame total-intensity normalization, one fixed mean-intensity scaling per record, and one-pixel Gaussian smoothing before normalization. Each ratio compares a thinned sequence with its own representation and matched native window. Absolute distances across representations have different meanings. Smoothing is a sensitivity operation, not validated noise correction. Source: Möller et al. [13,14], CC0; new analysis.

Figure S4 Original raw FLIM fields and aggregation



A and B show summed recorded photons with a shared display range of 0–35 counts per pixel; clipping affects display only. Scale bars are 50 μm . C shows normalized arrival histograms. D compares five declared masks under photon and equal-pixel weighting. The curves describe uncorrected first moments, not fitted lifetimes. Both selected files contain one physically retrieved acquisition frame, with additional frame references excluded. Source: Musimbi et al. [11,12], CC BY 4.0; new analysis and figure.

Figure S5 Participation and target load in the reconciled assay grids



Solid: same eligible ROI labels at all nine times. Dashed: eligible ROI labels available at each time.

Complete-time ROI counts (M0, IFN- γ , LPS, M2): CT26 = 5, 5, 5, 6; 4T1 = 2, 6, 3, 6.

Ratios of summed counts; lines guide the eye. No biological replicates, rates or optical pairing are inferred.

Panels a–c show CT26 and d–f show 4T1. Columns show $100 \times \Sigma P / \Sigma N$, $\Sigma E / \Sigma P$ and $\Sigma E / \Sigma N$, respectively, where N counts visible macrophages, P counts source-labelled uptake participants and E counts source-labelled engulfed targets. Colours preserve all four source condition labels. Solid curves restrict each context to the same eligible ROI labels at all nine acquisitions; dashed curves use eligible labels available at each acquisition. When curves overlap, the summaries coincide. Complete-time ROI counts for M0, IFN- γ , LPS and M2 are 5, 5, 5 and 6 for CT26 and 2, 6, 3 and 6 for 4T1. Axes share a scale within each metric column. Lines join observed time points without fitting; no uncertainty bands or biological replicates are inferred. Time zero is first acquisition after co-incubation. Numerators, denominators, ROI membership and alternative source values accompany the figure. These counts do not establish degradation, event rates or linkage to optical entries.