

# Selection and weighting change the interpretation of phagocyte uptake and optical imaging

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## Research Article

**Keywords:** phagocytosis, macrophage, fluorescence lifetime imaging, signal weighting, temporal sampling, secondary analysis

**Posted Date:** October 21st, 2026

**DOI:** <https://doi.org/10.21203/rs.3.rs-11442889/v1>

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**Additional Declarations:** The authors declare no competing interests.

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# 1 Selection and weighting change the interpretation of phagocyte uptake 2 and optical imaging

3 Short title: Selection and weighting in phagocyte imaging

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## 8 Abstract

9 The interpretation of phagocyte imaging depends on which cells are included, how detected signals  
10 are weighted and which temporal relationships are retained. We quantified these choices in three  
11 unlinked public resources. In macrophage assay tables, participation was the proportion of visible  
12 macrophages labelled as uptake participants. It increased from 24.1% to 37.5% using available  
13 regions but decreased from 46.7% to 37.5% using regions eligible throughout the series. Both  
14 inclusion rules began with the same participant count. In two raw macrophage fluorescence lifetime  
15 imaging microscopy fields, selecting bright pixels shifted the average recorded photon arrival to  
16 earlier times by 0.135 and 0.099 ns. These arrival summaries are not newly fitted fluorescence  
17 lifetimes. Both shifts exceeded conditional reference ranges that preserved pixel counts and the field  
18 arrival histogram, demonstrating an association between brightness and arrival under that reference.  
19 Overlapping deposited optical selections retained contrast directions without establishing a common  
20 direction across four macrophage–target contexts. In a supporting temporal comparison, three  
21 microglial movies retained their ordering by image variation in all 499 evaluated permutations at  
22 each interval, despite changes in accumulated variation. Including different regions therefore  
23 reversed a participation comparison, selecting bright pixels changed an optical summary, and  
24 disrupting temporal order preserved a ranking. These results guide denominator choice, reporting of  
25 signal weights and event-linked sampling criteria. They do not identify a shared functional marker,  
26 a metabolic cause or subsequent clearance capacity.

27 Keywords: phagocytosis; macrophage; fluorescence lifetime imaging; signal weighting; temporal  
28 sampling; secondary analysis

## 29 Introduction

30 A phagocyte must recognize, internalize and process material, and may then face another target.  
31 These stages create distinct biological questions. How many cells engage in uptake? How much  
32 target material is associated with the participating cells? Which cells can complete a later ingestion?  
33 Work on continual efferocytosis demonstrates that later clearance has specific requirements [1,2]. A  
34 measurement of material present at one acquisition therefore captures a different part of the process  
35 from a subsequent successful ingestion.  
36 Microscopy can address these questions through different observations. Cell and target counts  
37 describe participation and accumulated material. Fluorescence decay and intensity describe  
38 properties of the detected signal, while time-lapse records show how spatial organization changes.  
39 Endogenous fluorescence includes biochemical contributions that depend on context [3]. Optical  
40 features have distinguished uptake-associated or experimentally induced macrophage states in  
41 specific systems [4,5,6,7], and earlier work established effects of spatial and temporal sampling  
42 [8,9]. Each observable must therefore be interpreted in relation to the biological question it can  
43 answer; an optical difference alone does not establish a particular function.  
44 We examined three choices in the analysis of these observations. For uptake counts, which cells  
45 enter the denominator? For an optical mean, do all photons or all pixels contribute equally? For a  
46 movie summary, which relationships between successive images remain? We used macrophage  
47 optical tables and separate uptake-count tables [4,10], two raw macrophage fluorescence lifetime  
48 imaging microscopy (FLIM) fields [11,12], and three microglial confocal records [13,14]. Bess and  
49 colleagues already distinguished participation from an uptake index and examined context-  
50 dependent optical responses [4]. Möller and colleagues investigated sequential neuronal engulfment  
51 and its centrosome-related regulation [13]. We asked how selection and summarization change the  
52 interpretation of the deposited observations, without testing for a universal optical marker.

53 The resources cover different species, acquisitions and biological units; their measurements are not  
54 linked in the same cells. In the count tables, we compared participation after including different  
55 regions. In the raw FLIM fields, we measured the effect of selecting bright pixels and compared it  
56 with a conditional reference that preserved photon counts. We also reconciled overlapping optical  
57 exports to determine which contrast directions persisted, and permuted movie frames to test whether  
58 their ranking required the original order. Table 1 maps each question to its evidence and limits. The  
59 contribution is to quantify how these choices change the population or signal represented by a  
60 summary and, consequently, its interpretation.

61 **Table 1 Biological questions supported by the selected observations**

| Biological question                                         | Observation in these deposits                                                                                                                                      | Missing link or interpretive limit                                                                               |
|-------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|
| What does an uptake index combine?                          | Participation and aggregate target load have distinct denominators.<br><br>ROI inclusion reverses the M0/4T1 participation comparison.                             | No persistent cell identities, individual load distributions or evidence of degradation.                         |
| What does an uptake-associated optical difference describe? | Nine changed lists of active optical values retain contrast signs; three comparisons are identical.<br><br>No feature shares a direction across all four contexts. | No independent replication between exports; no linkage to the separate count records.                            |
| How is detected signal represented in a field mean?         | Bright-pixel selection shifts arrival by $-0.135$ and $-0.099$ ns beyond the specified count-conditioned references.                                               | Arrival moments are not fitted lifetimes; biological, background and instrument contributions remain unresolved. |

| Biological question                                                   | Observation in these deposits                                                                                  | Missing link or interpretive limit                                                                 |
|-----------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|
| Does a stable movie ranking retain the relevant temporal information? | Ranking persists in the 499 evaluated permutations per interval, while relative accumulated variation changes. | No event-rate calibration or function ranking; records differ in treatment and acquisition extent. |

## 62 Reading the optical measurements

63 FLIM records the delay between excitation and detection of fluorescence photons. A fluorescence  
64 lifetime describes how the signal decays over these delays. A mean across a field also depends on  
65 which pixels, photons or cells contribute and how much weight they receive. Table 2 distinguishes  
66 these quantities so that a change in one is not automatically interpreted as a change in another  
67 [3,11].

## 68 Table 2 Optical observables and their interpretation

| Quantity                          | What it represents                                                                                                                       | Unit and use here                                                                                                              |
|-----------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|
| Fitted component lifetime, $\tau$ | A decay time associated with one component of the chosen fit. Its biochemical assignment requires additional support.                    | Nanoseconds (ns). This is distinct from a mean derived from multiple fitted components; we do not fit new component lifetimes. |
| Fitted mean fluorescence lifetime | A summary derived from the fitted decay under the source averaging convention. Different weighting conventions can give different means. | ns. We reuse the deposited mean NADH lifetime and do not infer a particular weighting formula from the label alone.            |

| Quantity                       | What it represents                                                                                                                                                          | Unit and use here                                                                                                                                        |
|--------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|
| Component amplitudes and A1/A2 | Contributions of fitted components under the source convention; their ratio is not automatically a ratio of molecular concentrations.                                       | Dimensionless ratio. The deposited optical-table feature is the short-to-long component amplitude ratio.                                                 |
| Phasor coordinates             | Two normalized Fourier coordinates summarize the shape of a fluorescence decay and help display mixtures, without choosing a number of exponential components for each fit. | Dimensionless coordinates. The source study used phasor analysis of summed acquisitions; this secondary analysis does not calculate a new phasor result. |
| Mean recorded photon arrival   | The average recorded arrival coordinate under a specified photon or pixel weighting.<br><br>Instrument response, time origin and background affect that coordinate.         | ns. This is the quantity calculated for the two selected raw fields, not a fitted lifetime.                                                              |
| Optical redox ratio            | A ratio of detected fluorescence signals defined by the source measurement convention.                                                                                      | Dimensionless. Its unresolved numerator convention prevents assigning an oxidation direction here.                                                       |

69 The deposited A1/A2 label denotes the short-to-long fitted-component amplitude ratio, not

70 successive cargo exposures.

71 An optical comparison first establishes a difference in the measured quantity. Interpreting that

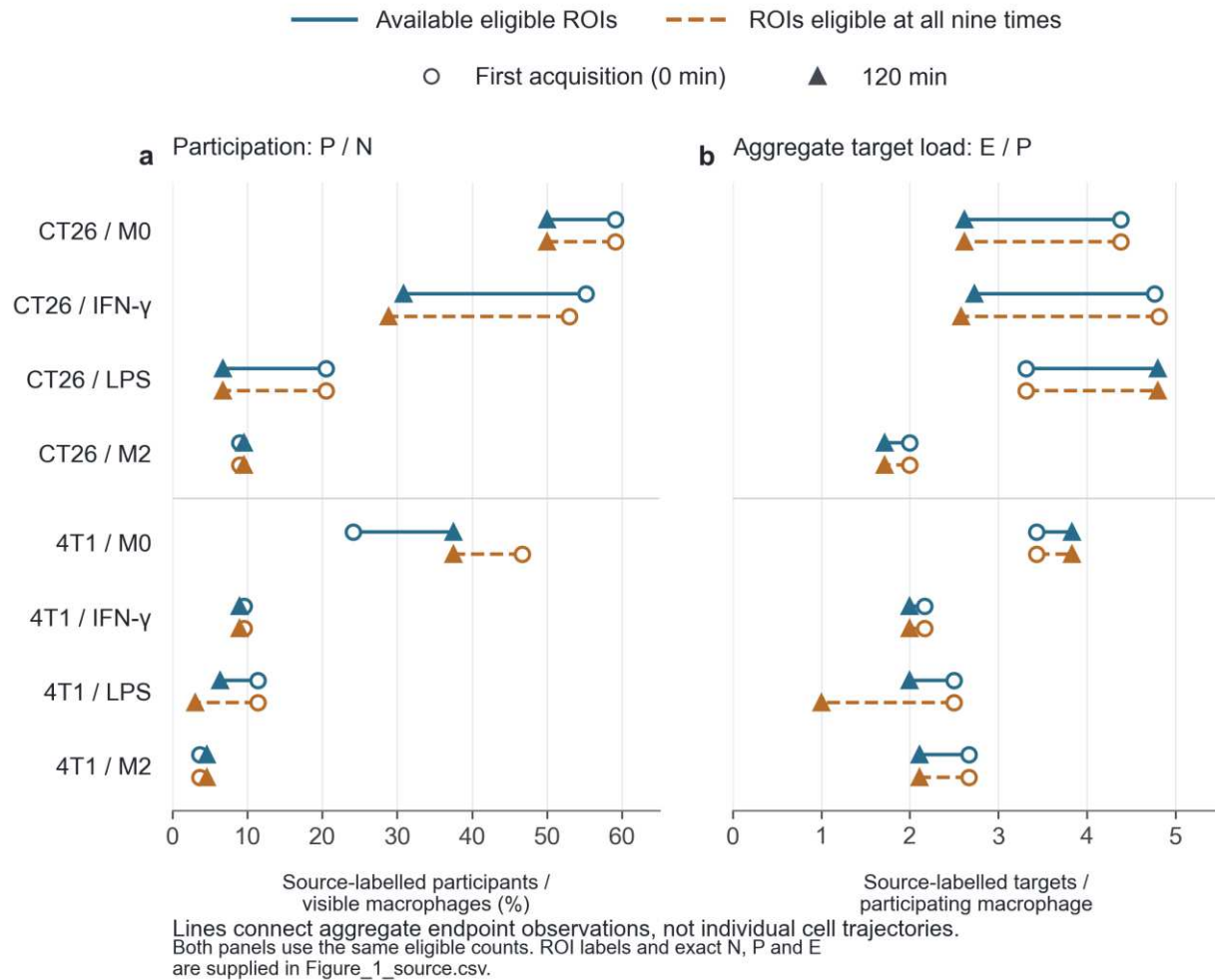
72 difference as metabolism, uptake capacity or future clearance requires observations linking the

73 quantity to the proposed process. The results below keep those interpretations separate.

## 74 Results

### 75 The same uptake index can represent different patterns of participation and target load

76 The Bess assay deposit records visible macrophages (N), macrophages labelled as participating in  
77 uptake (P), and targets labelled as engulfed (E). It covers murine RAW264.7 macrophages with  
78 CT26 or 4T1 tumour-cell targets, four source-labelled conditions and nine acquisition times. M0  
79 denotes medium only; the other labels are interferon- $\gamma$  (IFN- $\gamma$ ), lipopolysaccharide (LPS) and M2.  
80 These names identify source conditions, not universal macrophage classes. Time zero is the first  
81 acquisition after co-incubation, not a pre-contact baseline.  
82 For  $N > 0$ , participation is  $P/N$ . When  $P > 0$ , the aggregate labelled-target count per labelled  
83 participant is  $E/P$ ; we use “aggregate target load” for this ratio below. Their product  $E/N$  is the  
84 target count per visible macrophage. If  $P=0$  and  $E=0$ , participation and  $E/N$  are zero, but  $E/P$  is  
85 undefined. This factorization identifies the components of the index where both factors are defined;  
86 it is not a new biological law and does not recover individual-cell load distributions.  
87 We compared uptake summaries under two rules for including observed regions. After reconciling  
88 the copied worksheets, the primary criteria retained 373 of 432 region-of-interest (ROI), time and  
89 context records. Requiring each ROI label to be eligible at all nine acquisitions retained 38  
90 sequences, or 342 records. These labels do not establish independent cultures or continuous tracking  
91 of the same cells. Fig 1 shows participation and aggregate target load for all eight contexts under  
92 both rules; full trajectories remain in Supplementary Figure S5.



93

94 **Fig 1 Participation and aggregate target load describe different components of the uptake index.**

95 Endpoints are shown for all eight source contexts, comprising two tumour-cell targets and four macrophage condition  
 96 labels. (a) Participation is  $100 \times \Sigma P / \Sigma N$ ; (b) aggregate target load is  $\Sigma E / \Sigma P$ , where N counts visible macrophages, P  
 97 counts source-labelled uptake participants and E counts labelled engulfed targets. Open circles show the first acquisition  
 98 after co-incubation; filled triangles show 120 minutes. Both policies start from counts concordant across the three  
 99 copied sheets; first-sheet alternatives excluded by reconciliation remain in S1 Appendix. Blue solid lines use eligible  
 100 ROI labels available at each acquisition. Orange dashed lines restrict each context to ROI labels eligible at all nine  
 101 acquisitions. Lines connect aggregate observations, not individual-cell trajectories; their displacement is for readability.  
 102 Complete-time ROI counts in condition order M0, IFN-γ, LPS and M2 are 5, 5, 5 and 6 for CT26 and 2, 6, 3 and 6 for  
 103 4T1. Exact N/P/E counts and ROI membership for both policies accompany all 32 endpoints in Figure\_1\_source.csv.  
 104 No error bars or independent biological replication are inferred. Full time series are retained in Supplementary Figure  
 105 S5. Source data: [4,10].

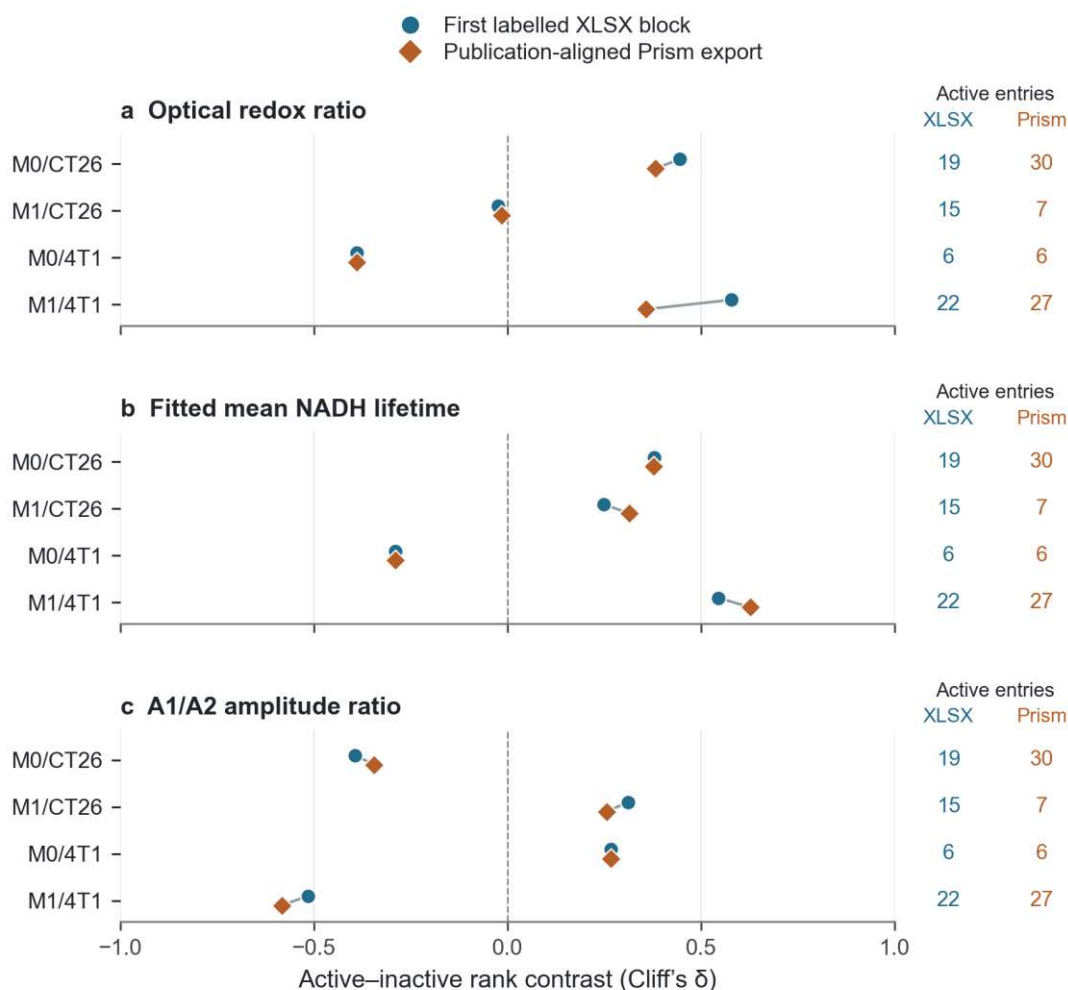
106 In the preselected first context, M0/CT26, the same five eligible ROI labels contained N=22, P=13  
107 and E=57 at the first acquisition, and N=26, P=13 and E=34 at 120 minutes. Participation changed  
108 from 59.1% to 50.0%, aggregate target load from 4.38 to 2.62, and their product from 2.59 to 1.31.  
109 The unchanged participant count does not show that the same cells participated at both times. These  
110 are counts present at each acquisition, not ingestion rates or evidence of degradation.  
111 Within the reconciled records, ROI inclusion reversed the direction of the M0/4T1 participation  
112 comparison. Participation increased from 24.1% to 37.5% using eligible ROI labels available at  
113 each acquisition, but decreased from 46.7% to 37.5% using the two labels eligible at all nine  
114 acquisitions. Both selections began with seven participants; their macrophage denominators were 29  
115 and 15. The reversal is attributable to the observed denominator change. Before cross-sheet  
116 reconciliation, the first-sheet grid additionally contained two ROI records absent from the other  
117 copies, giving N=60, P=7 and participation 11.7% at the first acquisition (S1 Appendix). Neither  
118 rule establishes the underlying biological response, and complete-time selection itself conditions  
119 inclusion on later availability.

## 120 **Uptake-associated optical contrasts have no common direction across the four contexts**

121 The source optical comparisons used M0 or IFN- $\gamma$ -treated macrophages, the latter labelled M1, with  
122 the same two target types. “Active” denoted detectable pHrodo-labelled target signal in an acidic  
123 compartment [4]. Detection depends on target material and its local pH; the “inactive” label does  
124 not establish inability to ingest. Deposited features were optical redox ratio, fitted mean NADH  
125 fluorescence lifetime and the short-to-long component amplitude ratio A1/A2. NADH denotes  
126 reduced nicotinamide adenine dinucleotide; the measured autofluorescence can also include  
127 contributions from NADPH. The redox numerator convention remained unresolved, so its direction  
128 was not assigned an oxidation meaning.

129 The first labelled XLSX block contained 182 optical values per feature; the publication-aligned  
130 Prism export contained 190. These entries are not verified independent cells or cultures. To  
131 compare active and inactive entries within a context, Cliff's delta counts the fraction of pairs in  
132 which the active value is higher and subtracts the fraction in which it is lower. This dimensionless  
133 balance ranges from  $-1$  to  $+1$ . Because biological grouping was not established for these entries, we  
134 did not calculate population tests or biological confidence intervals [15].

135 In context order M0/CT26, M1/CT26, M0/4T1 and M1/4T1, active counts were 19, 15, 6 and 22 in  
136 XLSX and 30, 7, 6 and 27 in Prism; every inactive group contained 30 entries. All 12 contrast signs  
137 agreed. However, the exports shared every inactive list of optical values and three active lists  
138 exactly. The other nine active lists differed, but their contrasts kept the same signs; three  
139 comparisons were identical by construction. Agreement therefore comes partly from reusing  
140 observations and does not provide independent replication. No feature had a common direction  
141 across all four contexts (Fig 2; Supplementary Table S2).



Each selection has 30 inactive entries per context. Counts are deposited entries, not verified biological replicates. Connectors pair selections; no uncertainty intervals are shown.

142

### 143 Fig 2 Optical contrast directions agree between overlapping selections but differ across contexts.

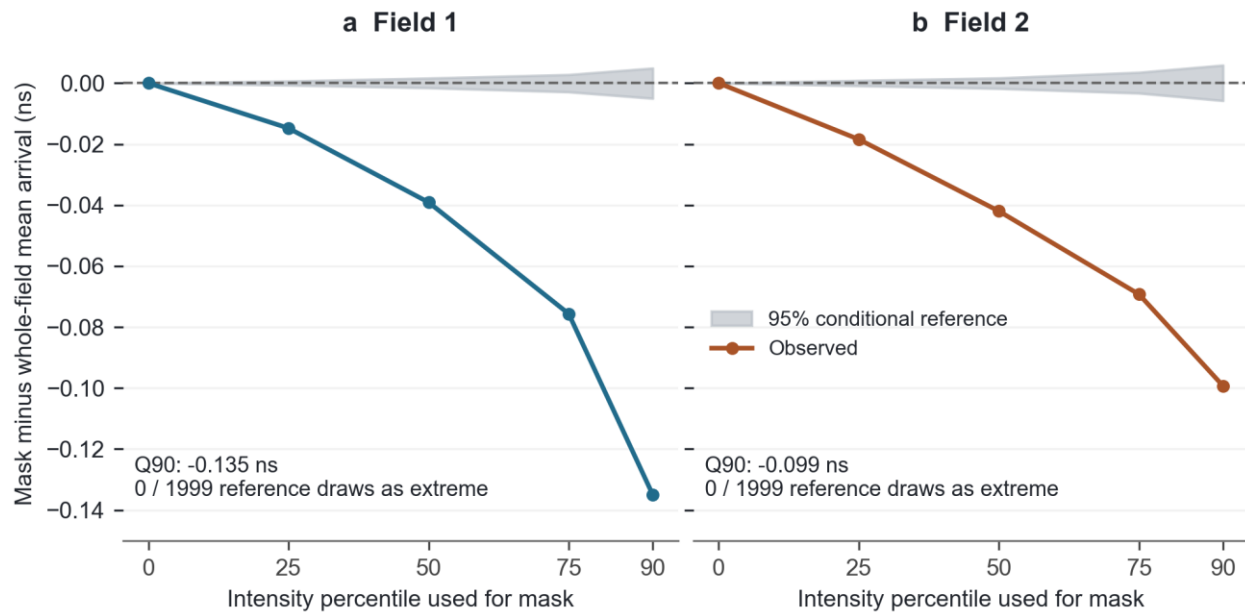
144 Active-inactive rank contrasts are shown for (a) deposited optical redox ratio, (b) fitted mean NADH lifetime and (c)  
 145 A1/A2 amplitude ratio. Blue circles denote the first labelled XLSX block; orange diamonds denote the publication-  
 146 aligned Prism export. Delta is the fraction of active-inactive pairs ordered higher minus the fraction ordered lower; ties  
 147 contribute zero. It is dimensionless and ranges from -1 to +1. Grey connectors pair estimates and are not uncertainty  
 148 intervals. Active and inactive are source labels based on the acid-sensitive pHrodo uptake signal [4]. Active-entry  
 149 counts are reported separately; all inactive groups contain 30 entries. Every inactive list of optical values and three  
 150 active lists are shared, so nine signs persist after a changed active list and three comparisons are identical. No feature  
 151 has one direction across all four contexts. These are finite deposited entries, not independent replication between  
 152 exports or verified biological units. Confidence intervals are not shown because biological grouping was not recovered;  
 153 entry-removal influence ranges remain in S1 Appendix. The redox numerator convention remains unresolved. Source  
 154 data: [4,10].

155 Redox delta ranged from  $-0.389$  to  $+0.579$  in XLSX and from  $-0.389$  to  $+0.382$  in Prism. For  
156 M1/4T1, the positive direction corresponded to  $+0.579$  and  $+0.358$ . For M1/CT26, contrasts were  
157 close to zero ( $-0.024$  and  $-0.014$ ); removing one XLSX entry at a time gave  $-0.098$  to  $+0.036$ . This  
158 influence range crosses zero and is not a confidence interval or evidence of a stable biological  
159 direction.

#### 160 **Brighter regions contribute shorter mean recorded arrival in the two selected fields**

161 A photon-weighted mean lets brighter regions contribute more because they contain more detected  
162 photons. An equal-pixel mean gives each pixel with detected photons the same weight. We asked  
163 whether selecting bright pixels shifted mean recorded photon arrival beyond what a specified  
164 redistribution would produce. In this reference, arrival coordinates were redistributed among photon  
165 positions while each pixel retained its photon count and the whole field retained its arrival  
166 histogram.

167 The two porcine macrophage control fields contained 2,790,695 and 1,801,514 photons. At masks  
168 retaining positive pixels at or above the 90th intensity percentile, mean arrival changed from 3.632  
169 to 3.497 ns and from 3.724 to 3.625 ns. The masks retained 28.445% and 29.825% of photons.  
170 Shifts of  $-0.135$  and  $-0.099$  ns lay outside the pointwise central 95% conditional ranges of  $-0.005$   
171 to  $+0.005$  ns and  $-0.006$  to  $+0.006$  ns (Fig 3; Supplementary Table S5). None of 1,999 realizations  
172 per mask reached the observed absolute shift; this finite result does not imply zero probability.  
173 Across all positive pixels, equal-pixel means exceeded photon-weighted means by 0.105 and 0.127  
174 ns.



175

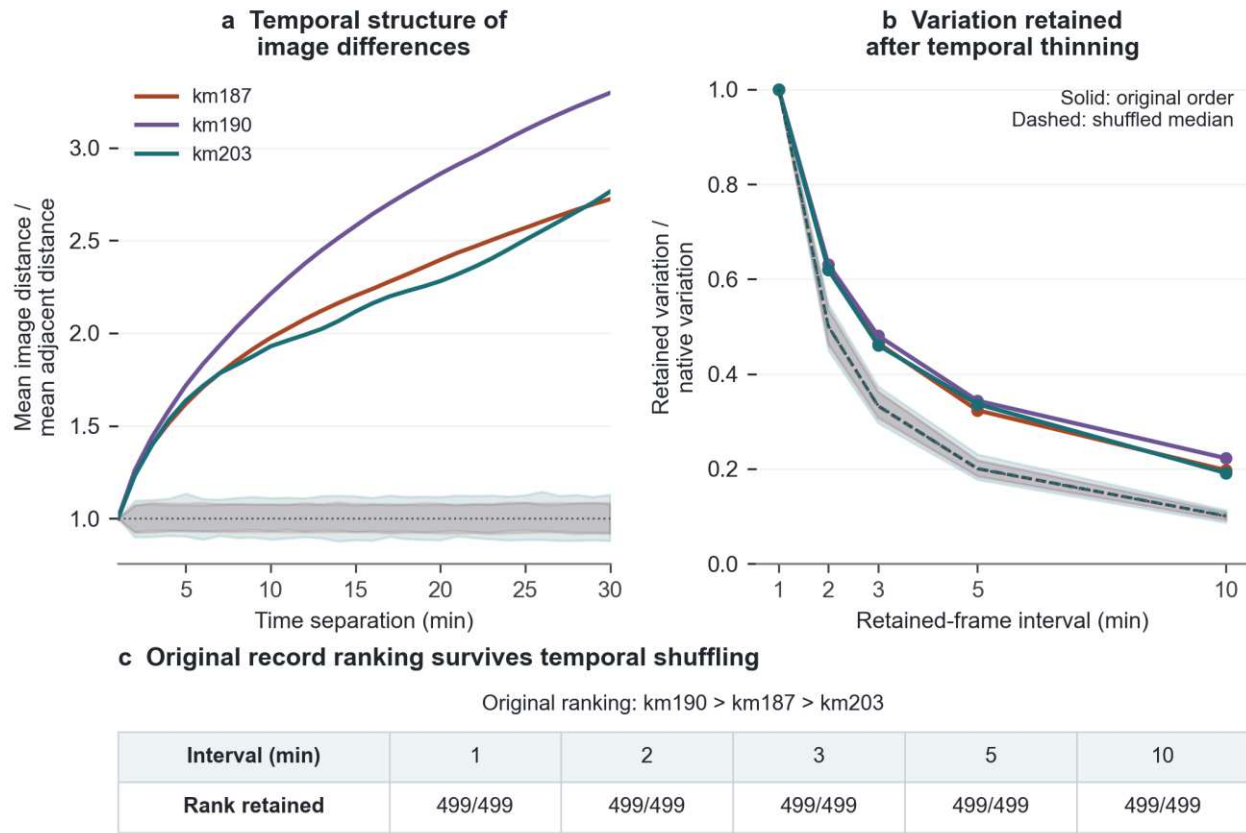
176 **Fig 3 Bright-pixel selection shifts recorded arrival beyond a count-conditioned reference.**

177 (a,b) Mask-minus-field photon-weighted mean arrival coordinates for the first physical acquisition frame of each  
 178 porcine macrophage field. Masks retain positive pixels at or above each intensity percentile, including ties. Coloured  
 179 points and lines show observed shifts; grey shading gives pointwise central 95% ranges from 1,999 multivariate  
 180 hypergeometric realizations per nontrivial mask. The reference preserves pixel counts and the field arrival histogram  
 181 while removing association between arrival coordinates and pixel identity. At percentile zero, the mask is the entire  
 182 positive-pixel field and the shift is zero. Q90 annotations report the observed shift and reference realizations at least as  
 183 extreme in absolute magnitude. Ranges are conditional on these arrays, not biological confidence intervals or a  
 184 complete physical noise model. Arrival coordinates are uncorrected for instrument response, background and time zero;  
 185 they are not fitted lifetimes. Masks are not cell segmentations. This analysis does not reproduce the source study's  
 186 processing or assign a metabolic cause. Source data: [11,12].

187 Brightness and recorded arrival were therefore associated within these fields under the specified  
188 reference. The result does not distinguish biological heterogeneity from heterogeneous background  
189 or spatial instrument effects. No instrument-response, background or time-zero correction was  
190 applied, and masks were not cell segmentations. These moments describe recorded arrival  
191 coordinates, not fitted lifetimes or metabolic rates. They characterize the first physical frame  
192 selected here, rather than reproducing the source study's phasor analysis of summed acquisitions  
193 [11]. These results support reporting both the weighting rule and the fraction of signal retained.  
194 They do not identify an optimal threshold or a metabolic cause.

#### 195 **Movie ordering survives loss of temporal sequence, while accumulated variation changes**

196 We asked whether the ranking of movies by image variation depended on their original frame order.  
197 Image variation per minute sums distances between consecutive frames after normalizing their  
198 intensities and divides by elapsed minutes. It describes image change, not an engulfment rate. The  
199 three zebrafish microglial records differ in acquisition extent, duration and source treatment, so this  
200 ranking cannot establish a hierarchy of intrinsic cell function.  
201 The ordering  $km_{190} > km_{187} > km_{203}$  remained unchanged in all 499 evaluated permutation  
202 iterations at each of five sampling intervals (Fig 4). Each permutation preserved complete images  
203 and all pairwise distances while removing the original sequence. This finite result concerns the  
204 medians over starting offsets; it is not a population probability of 100%.



Three zebrafish microglia records; 499 temporal permutations per record.  
Shading: central 95% of frame permutations, not biological confidence intervals.

205

**Fig 4 Temporal organization affects accumulated variation while the movie ranking survives permutation.** Three processed microglial records were analysed after normalizing each frame by total intensity. (a) Mean image distance at each lag, divided by the mean lag-one distance of that same original or permuted sequence. Solid curves show original order; shading gives pointwise central 95% ranges from 499 frame permutations per record. Image distance is half the summed absolute pixel differences. (b) Retained-to-native accumulated distance  $R$  over matched endpoints: original offset medians are solid, permutation medians dashed, and central 95% ranges shaded. The dotted  $1/k$  curve is a reference scale, not the expected ratio;  $R \leq 1$  follows from the triangle inequality. (c) The ranking km190 > km187 > km203 recurs in all 499 evaluated iterations at each interval. Complete images and all pairwise distances are preserved; temporal order is removed. These finite results do not establish a probability of 100% or preservation of engulfment events. Records contain 82, 120 and 60 frames for km187, km190 and km203; their treatment, extent and duration differ. The one-minute cadence is metadata-confirmed for km203 and inventory-derived for the others. Source data: [13,14].

Temporal organization nevertheless affected accumulated variation. Retention  $R$  compares accumulated image distance after thinning with the native sum over matched endpoints. The triangle inequality guarantees  $R \leq 1$ . At ten minutes, original offset medians were 0.197, 0.222 and 0.191 for km187, km190 and km203. Permutation medians were 0.100, 0.100 and 0.101, with central 95% ranges of 0.092–0.108, 0.093–0.109 and 0.087–0.113 (Supplementary Table S6). Original adjacent-frame distances were, on average, 0.405, 0.302 and 0.442 times each record's mean over all image pairs.

Overall image dispersion helps explain why a ranking could persist. Mean all-pair distances were 0.0702, 0.1429 and 0.0459, already ordered km190 > km187 > km203. Under uniform permutation, the expected variation per minute for a fixed offset is the all-pair mean distance divided by the sampling interval. This expectation does not determine the median over offsets, expected retention or the ranking of every realization. The distinction matters: the ordering survived, while the descriptor's relation to temporal sequence changed. Drift, bleaching, structured noise and biological motion were not separated. Choosing a coarser acquisition interval therefore requires a criterion tied to the events or temporal relationship of interest.

## **Discussion**

### **Selection changes the biological comparison represented by a summary**

Selection changes what a comparison represents. Including different regions reverses the M0/4T1 participation comparison while retaining the same initial participant count. Selecting bright pixels changes mean recorded arrival in both raw fields beyond the specified count-preserving conditional references. The first result depends on which macrophages enter the uptake comparison; the second depends on which detected photons dominate the optical mean. Both quantify an analytical choice that must remain visible when interpreting a change, without identifying a new biological

241 mechanism. The optical-export and movie analyses show a complementary result: a contrast  
242 direction or ranking can persist even when observations are shared or temporal information is  
243 altered.

244 For uptake, Bess and colleagues already reported participation and an uptake index [4]. Factoring  
245 the index into participation and target load is an algebraic identity; the additional result here is the  
246 measured sensitivity to region inclusion in the deposited counts. A lower target count per visible  
247 macrophage can reflect a lower proportion of participating macrophages ( $P/N$ ), fewer labelled  
248 targets per participant ( $E/P$ ), or both, where the ratios are defined. The M0/CT26 example separates  
249 these components; the M0/4T1 example shows that equal initial participant counts can yield  
250 opposite participation comparisons when the selected macrophage denominator changes. Neither  
251 inclusion rule establishes the true biological trajectory: complete-time eligibility also conditions  
252 selection on later availability. Tracked identities would be needed to distinguish changes in cell  
253 participation from migration or field composition; tracking targets together with measurements of  
254 processing would help separate continued presence, loss of detectability and degradation. These are  
255 different biological explanations of the same aggregate observations.

256 For the deposited optical features, Bess and colleagues already described context-dependent uptake-  
257 associated responses and modest correlations with phagocytic metrics [4]. The present  
258 reconciliation identifies which deposited values support the contrasts, how much of the agreement  
259 comes from shared entries, and where a near-zero contrast is sensitive to one entry. It adds a  
260 quantitative account of sensitivity and overlap, rather than the first observation of contextual  
261 specificity. Fitted optical features also share measurement components, so they do not provide  
262 independent biological confirmations. Linking optical entries to the count observations would be  
263 necessary before asking whether a particular optical state describes participation, target load or a  
264 different aspect of uptake in these deposits.

## 265 **Signal weighting is part of the observable**

266 The raw FLIM analysis quantifies an association between brightness and recorded arrival within the  
267 selected fields. Giving each detected photon equal weight emphasizes bright regions; giving each  
268 positive pixel equal weight emphasizes spatial locations regardless of brightness. These averages  
269 answer different questions about the same signal. Their difference, together with the fraction of  
270 photons retained by a mask, shows how the selection rule affects the summary. The conditional  
271 reference asks what mask–field difference would arise if recorded arrival coordinates were  
272 redistributed among photon positions, while each pixel retained its photon count and the whole field  
273 retained its arrival histogram. This comparison does not assign a molecular source to the  
274 association.

275 Photon weighting and background-sensitive interpretation have established precedents. Coucke and  
276 colleagues used photon-weighted phasor averages and explicit background contributions in particle-  
277 based FLIM measurements of Förster resonance energy transfer [16]. Scipioni and colleagues  
278 demonstrated how background contributions can bias conventional FLIM measurements at low  
279 fluorophore expression and how spectral separation can address them [17]. These studies concern  
280 different optical summaries and systems; they establish why weighting and mixtures require  
281 attention, not the cause of the shifts in our macrophage fields. The additional evidence here is the  
282 magnitude of the selection effect in these two raw records and its comparison with an explicitly  
283 constrained reference, rather than a new principle of fluorescence averaging.

284 The observed association does not identify its cause. A common additive shift of the same arrival  
285 coordinates cancels from a mask–field difference when weights and selection remain fixed.

286 Spatially varying contributions need not cancel: a changing mixture of signal and background with  
287 different mean arrivals can produce brightness–arrival association without changing the signal  
288 component’s mean. This is an illustrative counterexample, not a fitted explanation of these fields.

289 Biological heterogeneity, background composition and spatial instrument effects remain  
290 alternatives. A next discriminating comparison would retain a common acquisition and cell  
291 definition while examining those contributions with the relevant corrections; repeating a threshold  
292 comparison alone would not resolve them.

293 Our first-frame arrival moments also differ from the original summed-acquisition phasor processing  
294 [11]. The result should guide the definition and reporting of an optical summary, rather than be used  
295 as a direct test of that source study's metabolic conclusions. No optimal intensity threshold follows  
296 from the conditional reference.

### 297 **Temporal sampling must preserve the relationship of interest**

298 The confocal comparison shows why preserving a ranking is not enough to preserve temporal  
299 information. Overall image dispersion already ordered the three records, helping explain why their  
300 ranking survived permutation. Original neighbouring frames were nevertheless more similar than  
301 arbitrary pairs, and permutation changed relative accumulated variation. A stable ranking therefore  
302 does not certify that a summary retains cup formation, closure or relationships between successive  
303 ingestions. Comes and colleagues previously quantified how spatial and temporal resolution affect  
304 measurements of cell–cell interactions [8]. The additional observation here is that this particular  
305 ordering survives temporal disruption, not the general discovery that sampling can lose information.  
306 We selected this descriptor for the secondary analysis; its behaviour does not overturn the event-  
307 based and perturbational evidence in the source microglial study [13].

308 For a biological comparison, the acquisition interval should preserve a defined event or temporal  
309 relation. One candidate question is whether the association between the location of a new  
310 phagocytic cup and its later closure differs according to whether the last observed ingestion  
311 occurred at a branch tip or the cell body. This would require history defined before the current  
312 attempt, comparable observation of initiation and closure, linked cell and target identities, and

313 treatment of reattempts and lost follow-up. Möller and colleagues already investigated sequential  
314 ingestion and centrosome-related dynamics [13]; the novelty of a further contrast must be judged  
315 against that work. The current analysis specifies the information needed, without claiming that this  
316 candidate relationship has been demonstrated.

### 317 **Connecting scales through measured function**

318 To ask whether a subcellular optical measurement anticipates a later ingestion, the signal and event  
319 must be linked in the same tracked cell. For a proposed subcellular extension, the region would also  
320 require validated identity and separation of host-cell signal from ingested cargo. Shared chemical  
321 contrast or a fluorescence lifetime alone would not identify organelle activity. The biological  
322 question is whether that measurement adds useful information beyond prior uptake, target  
323 availability and observation time. Counts with explicit denominators and follow-up of cohort  
324 membership would then locate those cellular events within a defined population response.  
325 Participation, accumulated target material and later successful ingestions remain different outcomes.  
326 A predictive gain over a prespecified simpler description would warrant further comparison of  
327 candidate explanations; it would not by itself identify metabolism, recovery or a causal mechanism.  
328 Combining modalities or associating measurements from separate datasets cannot supply the  
329 missing linkage.

330 The present three resources are unlinked and do not validate a multiscale mechanism, an optical  
331 biomarker or a clinical prediction. Their immediate contribution is to make selection, weighting and  
332 temporal information explicit before a cross-level relationship is interpreted. If a linked optical  
333 description fails to add prespecified useful information with adequate precision in independent  
334 biological units, its incremental value would be limited for that outcome and setting. An imprecise  
335 comparison would remain inconclusive. Conversely, predictive improvement would still need  
336 scrutiny for selection, opportunity and acquisition effects.

### 337 **Scope of the inference**

338 These cases were selected pragmatically, and conditional controls followed initial descriptive  
339 analyses. The three resources were not sampled to estimate how often these sensitivities occur  
340 across phagocytes. Independent biological grouping, acquisition effects and links between optical  
341 entries and functional counts remain unresolved. Conditional reference ranges describe specified  
342 rearrangements of finite observed arrays, not uncertainty among animals or cultures. Algebraic  
343 identities explain how a summary is constructed; they do not constitute a biological discovery.  
344 Computational agreement establishes reproducibility within its documented inputs and checks, not  
345 biological validation. Within this scope, the results support three practical choices: retain the counts  
346 and membership behind a participation comparison, report the weights and signal fraction  
347 represented by an optical mean, and assess temporal sampling against the event or relationship of  
348 interest. These choices make the connection from measurement to biological interpretation testable.

### 349 **Materials and methods**

350 This secondary study compared alternative region inclusion, optical signal selection and temporal  
351 ordering within three public resources. The analyses retained the original observation units and did  
352 not pool species, fields or movies into a common biological sample. The methods below specify the  
353 selections, summaries and conditional references that produced the reported results; extended  
354 derivations and complete outputs are retained in S1 Appendix and S2 Data.

### 355 **Study design and analysis history**

356 Selection was pragmatic, based on biological relevance, accessible numerical inputs, metadata and  
357 feasible local execution. Initial module plans preceded effect calculations but followed knowledge  
358 of the publications. Source-block reconciliation and pooling decomposition were subsequent

359 descriptive audits. The conditional controls reported here were designed after the initial descriptive  
360 analyses, in response to their results and methodological criticism; their plans preceded their  
361 calculations. None was preregistered. The count reconciliation added in V6 followed the earlier  
362 dataset audit; its exploratory plan fixed eligibility and summaries before the new calculations.  
363 Optical rows were not linked to the assay counts.

#### 364 **Data sources and observation units**

365 Bess data came from Figshare project 163321 [4,10]: murine RAW264.7 macrophages under  
366 source-labelled M0 medium-only or M1 interferon- $\gamma$  conditions, with CT26 or 4T1 tumour-cell  
367 targets. The source active label used a pHrodo uptake signal. Features were deposited optical redox  
368 ratio, fitted mean NADH (reduced nicotinamide adenine dinucleotide) fluorescence lifetime in  
369 nanoseconds and short-to-long amplitude ratio A1/A2. Autofluorescence can include both NADH  
370 and NADPH, its phosphorylated counterpart; we retained the source feature name without  
371 separating these cofactors. The redox numerator convention remained unresolved, so no oxidation  
372 direction was assigned. The two fitted features are not independent biological corroborations.  
373 Two raw control fields of porcine PAM 3D4/31 macrophages came from Zenodo 14330511 [11,12].  
374 Three zebrafish microglial confocal records came from BioImage Archive S-BIAD564 [13,14]. All  
375 component-3 tagged image file format (TIFF) candidates with one lyn-tagRFPT channel, a reported  
376 60-second interval, at least 30 frames and size  $\leq 125$  MB were included. Source projections and  
377 crops were retained. The S1 Appendix documents selection, units and full results.

#### 378 **Optical table extraction and contrasts**

379 The first explicitly labelled XLSX block was selected before the historical effect calculation. We  
380 now display it and the publication-aligned Prism export symmetrically, preserving the analysis  
381 history and all numerical results. Duplicate blocks were not concatenated. For  $n_{\text{active}} \times n_{\text{inactive}}$

value pairs within a context, Cliff's delta was (pairs with the active value higher – pairs with the active value lower)/(n\_active × n\_inactive); ties contributed zero. The full set of 12 context-by-feature contrasts is retained for each selection.

The first labelled block and the publication-aligned Prism export were treated as alternative analytical populations. We retained every contextual contrast and did not pair features across rows without persistent identities. Leave-one-entry-out ranges describe influence, not confidence intervals. For the first XLSX block, the pooled contrast was decomposed into within-context and cross-context pairs by exact enumeration. Supplementary Tables S1–S3 give the observation units, full contrasts and weighting decomposition.

### **Assay counts and denominator reconciliation**

N denotes the visible-macrophage count, P the source-labelled participant count and E the source-labelled engulfed-target count. These assay symbols are separate from the photon and pixel symbols in the arrival calculation below. The assay condition labels are M0, IFN- $\gamma$ , lipopolysaccharide (LPS) and M2; the optical contrasts use only M0 and the IFN- $\gamma$  condition labelled M1. These are retained source labels, not universal macrophage classes. The source grid covers two targets, these four conditions, six ROI labels and nine nominal times, 0–120 minutes at 15-minute intervals. Time zero is the first acquisition after co-incubation, not a pre-contact baseline.

An exploratory plan for the count reconstruction was recorded after the earlier source audit and before calculating its trajectories. We extracted the first raw-count grid from each of six assay worksheets and preserved all copies and cell coordinates. A common record required exact agreement across the three metric sheets, nonnegative integer counts,  $N > 0$ ,  $P \leq N$ ,  $E \geq P$  and  $E = 0$  when  $P = 0$ . The  $E \geq P$  criterion was a conservative requirement for interpreting E/P as distinct targets per participant, not a declaration that other definitions are biologically impossible. We retained all source alternatives without imputation. At each target, condition and time, we calculated ratios of

406 summed counts and checked  $\frac{E}{N} = \left(\frac{P}{N}\right)\left(\frac{E}{P}\right)$  with exact rational arithmetic wherever defined. Available-  
407 ROI summaries were compared with summaries restricted to ROI labels eligible at all nine  
408 acquisitions. A sum counts objects in the selected regions at that acquisition; it does not establish  
409 unique tracked cells. The complete-time restriction conditions inclusion on later availability and is a  
410 sensitivity analysis, not a corrected biological population. No counts were linked to individual  
411 optical entries. A separate, explicitly subsequent sensitivity retained  $E < P$  observations as arithmetic  
412 ratios without asserting the load interpretation.

### 413 **Raw photon aggregation and its conditional reference**

414 Each physical FLIM file contained photon counts at 227 recorded arrival coordinates for each of  
415  $512 \times 512$  pixels. Custom metadata identified a 0.054991 ns coordinate increment despite a generic  
416 Open Microscopy Environment (OME) Z label. These coordinates represent recorded delays after  
417 excitation, not fitted fluorescence lifetimes. Only the first physical acquisition frame per field was  
418 analysed; four referenced external frames per series were not retrieved or zero-filled. Intensity was  
419 the count summed over arrival bins. Masks retained positive pixels at or above percentiles 0, 25, 50,  
420 75 and 90, including ties.

421 Photon-weighted means average recorded arrival coordinates over detected photons. Equal-pixel  
422 means first average within each positive pixel and then weight pixels equally. These are different  
423 estimands; their difference is exactly related to the covariance of intensity and pixel mean arrival  
424 (S1 Appendix). This algebraic identity does not by itself identify meaningful heterogeneity.

425 We therefore used a conditional reference that preserves every pixel count and the complete field  
426 arrival histogram, but uniformly reassigns arrival coordinates among photon positions. Count-  
427 defined masks remain fixed. For each nontrivial mask, 1,999 multivariate hypergeometric draws

reproduce its finite-population arrival histogram under this reference. Central 95% ranges are pointwise conditional reference ranges, not biological confidence intervals. The arrival reference used seed 24092026 and NumPy 2.3.5 multivariate hypergeometric sampling, method marginals. Each mask was simulated separately. For M photon positions selected from N observed field coordinates with sample variance  $S^2$ , the variance of the mask–field mean difference is  $S^2(\frac{1}{M} - \frac{1}{N})$ . For P positive pixels with counts  $I_p$ , the equal-pixel minus photon-weighted mean has conditional variance  $S^2[\frac{\sum p \frac{1}{I_p}}{P^2} - \frac{1}{N}]$ . The assumptions, derivations and independent arithmetic checks are given in S1 Appendix. Quantiles are pointwise conditional references, not biological confidence intervals or simultaneous bands.

### **Confocal variation and temporal permutation**

The confocal records contained 60, 82 and 120 frames. ImageJ metadata confirmed a one-minute interval for km203; timing for km187 and km190 relied on the source inventory. Time zero was the first stored frame. Each frame was divided by its total intensity. Distance between frames was half the sum of absolute pixel differences. Summed consecutive distances divided by elapsed minutes defined image variation per minute, an image descriptor rather than a cellular activity rate. We retained frames every 1, 2, 3, 5 or 10 minutes at every starting offset. Native and thinned sequences shared endpoints. Retention R measures how much accumulated image change remains when intermediate frames are omitted: it is the coarse accumulated distance divided by the native accumulated distance over that same window. The triangle inequality guarantees  $R \leq 1$ . For interval multiplier k, kR equals mean retained-edge distance divided by mean native-edge distance; this identity separates edge length from the number of sampled edges. The temporal control independently permuted each record's frame order 499 times, preserving complete images and all pairwise distances. Each iteration repeated thinning at every offset; offset

451 medians summarized each record. For a fixed matched window,  $1/k$  is the ratio of the expected  
452 coarse and native accumulated distances; it is a reference scale, not the expected retention.  
453 Full-resolution pairwise distances were calculated between total-intensity-normalized frames. Seed  
454 20260924 and the PCG64 generator defined the 499 independent permutations per record. All  
455 offsets were evaluated at each interval; medians over offsets were calculated before summarizing  
456 permutations or ordering records. Original metrics were checked against the initial implementation  
457 at an absolute tolerance of  $10^{-7}$ . All permutation and phase outputs are supplied, including the  
458 distinction between original all-offset sensitivity and the median-based permutation analysis. In Fig  
459 4a, distances at each lag are divided by the mean lag-one distance of the same original or permuted  
460 sequence.

#### 461 **Computational verification**

462 Sources, analysis plans, numerical outputs and prior checks are preserved in the accompanying  
463 package. Historical checks include a separate XML extraction route and rational arithmetic for  
464 optical tables, exact illustrative checks of photon variance, a replay of the two conditional controls,  
465 and an independent reconciliation of assay counts. The confocal replay began from three TIFFs; the  
466 arrival replay began from two derived NPZ arrays. Their receipts document their dates, inputs and  
467 scope; they are not presented as new executions in this revision.

468 During the V8 editorial revision, a separate script recomputed 24 reported optical deltas from  
469 preserved extracted CSVs using rational pair comparisons, checked 432 aggregate N/P/E sums  
470 across 144 count summaries against the reconciled records, and counted 2,495 recorded  
471 permutation-ordering rows. The checked values agreed with the retained results. This bounded  
472 verification did not repeat source XLSX extraction, TIFF decoding or simulations. Fig 1 was  
473 generated from the preserved count CSV; it displays all eight contexts and both inclusion rules at  
474 the first and 120-minute acquisitions. Its 32 endpoint records retain exact numerator, denominator

475 and ROI membership. Choosing this display followed the existing results and adds no inferential  
476 test.

477 Computational agreement does not certify functional labels, independent biological units or  
478 experimental replication. Automated reviews were non-human; interpretation and final approval  
479 remain the responsibility of the human author. This editorial adaptation did not alter the retained  
480 data, model fits, simulations or scientific results. Its numerical checks and figure-format exports are  
481 documented separately in the package receipt.

## 482 **Use of artificial intelligence**

483 OpenAI Codex assisted source retrieval, programming, execution and numerical checking,  
484 manuscript drafting, editorial restructuring, figure preparation and methodological criticism. The  
485 materials distinguish replay of existing implementations, separate arithmetic checks and automated  
486 reviews. No synthetic microscopy image or fabricated experimental observation was introduced.  
487 Agent reviews are non-human and do not constitute peer review. Responsibility for scientific  
488 decisions, interpretation and final approval rests with the named human author.

## 489 **Source reconciliation details**

490 The source audit also identified 54 count formulas beneath 45-minute labels that referenced 30-  
491 minute grids. We reconstructed counts from the raw grids. This localized discrepancy was not  
492 assigned to the published results or uniformly to Prism trajectories; S1 Appendix preserves the  
493 exact cells and a counterexample to that extrapolation.

494 The nine changed lists of active optical values matched the second spreadsheet block in order.  
495 Prism reproduced all 12 explicitly reported optical mean–dispersion pairs after rounding, compared  
496 with four for the first XLSX block. This supports the term publication-aligned without recovering  
497 the selection rules. Reconciling the exports shows which values were compared and how the

498 contrasts change. It does not provide independent biological replication or establish a common  
499 direction across macrophage–target contexts. Supplementary Table S3 decomposes pooling for the  
500 first XLSX block only.

## 501 **Declarations and availability**

## 502 **Author contributions**

503 Conceptualization, methodology, software, formal analysis, visualization, writing – original draft,  
504 and writing – review & editing: Elkin Navarro Quiroz. The author approved the final manuscript  
505 and takes responsibility for the work.

## 506 **Competing interests**

507 The author declares no competing interests.

## 508 **Ethics and data reuse**

509 This secondary analysis used publicly deposited numerical data and microscopy files. It introduced  
510 no new participant recruitment, specimen collection or animal experiments. Original acquisition  
511 approvals are reported by the source studies.

## 512 **Code availability**

513 S2 Data contains the public numerical package derived from V7 and the V8 figure, verification  
514 code, source tables and instructions, within a journal-specific numerical wrapper. The V8 scripts  
515 reproduce the count display and bounded numerical checks from included inputs. The historical V7  
516 package supplies analysis settings, source manifests, dependency records and entry points for count  
517 reconstruction, the conditional controls and mathematical clarification checks. Its compact archive  
518 contains derived FLIM arrays but omits five original microscopy TIFFs. The complete local review

519 bundle preserves those TIFFs and instructions for deeper replay. Each receipt distinguishes newly  
520 executed checks from historical ones. The original analysis and verification code is released under  
521 the MIT License. Source datasets and third-party materials retain their original licences; the MIT  
522 licence does not replace them. The licence and scope notice are included in S2 Data.

## 523 **Data availability**

524 Original data are available from Figshare project 163321, Zenodo 14330511 and BioImage Archive  
525 S-BIAD564 [10,12,14]. Figshare and Zenodo sources carry CC BY 4.0 licences; S-BIAD564 states  
526 CC0. S1 Appendix provides methods, seven tables and five supplementary figures. S2 Data  
527 contains deposited numerical sources, derived arrays, complete analysis outputs, source-file  
528 manifests and code. The exact five selected microscopy TIFFs remain available from the cited  
529 repositories and in the separate full local review bundle. Their acquisition routes and checksums are  
530 recorded. The eight additional FLIM acquisition files referenced by the selected two series were not  
531 analysed or treated as empty observations.

## 532 **Acknowledgments**

533 The author thanks the Asociación Colombiana de Inmunología (ACOI) and the investigators who  
534 made the source data publicly available.

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## 588 **Supporting information captions**

589 S1 Appendix. Extended methods, seven tables and five supplementary figures. Scientific  
590 supplement aligned 6 October 2026; numerical content retained. Citation numbers in this appendix  
591 refer to the main article’s reference list. Historical texts archived within S2 Data retain their original  
592 numbering and edition maps.

593 S2 Data. Numerical sources, code, derived arrays, analysis outputs and portable verification. The  
594 public package preserves numerical analysis inputs, code, derived arrays and outputs while omitting  
595 historical administrative author and declaration metadata. Its provenance record and fresh integrity  
596 and selected arithmetic receipt identify that curation. Five original microscopy TIFFs are identified  
597 by repository routes and checksums and are not included; the eight additional FLIM acquisition  
598 files were not analysed. Original code is licensed under MIT; source materials retain their licences.  
599 Preparation does not itself certify external posting or biological validation.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [S1Appendix.pdf](#)
- [S2Data.zip](#)