Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

Rapid Time-Lapse 3D Oxygen Tension Measurement within Hydrogels Using Widefield Frequency Domain Fluorescence Lifetime Imaging Microscopy (FD-FLIM) and Image Segmentation

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Algorithm Flowchart

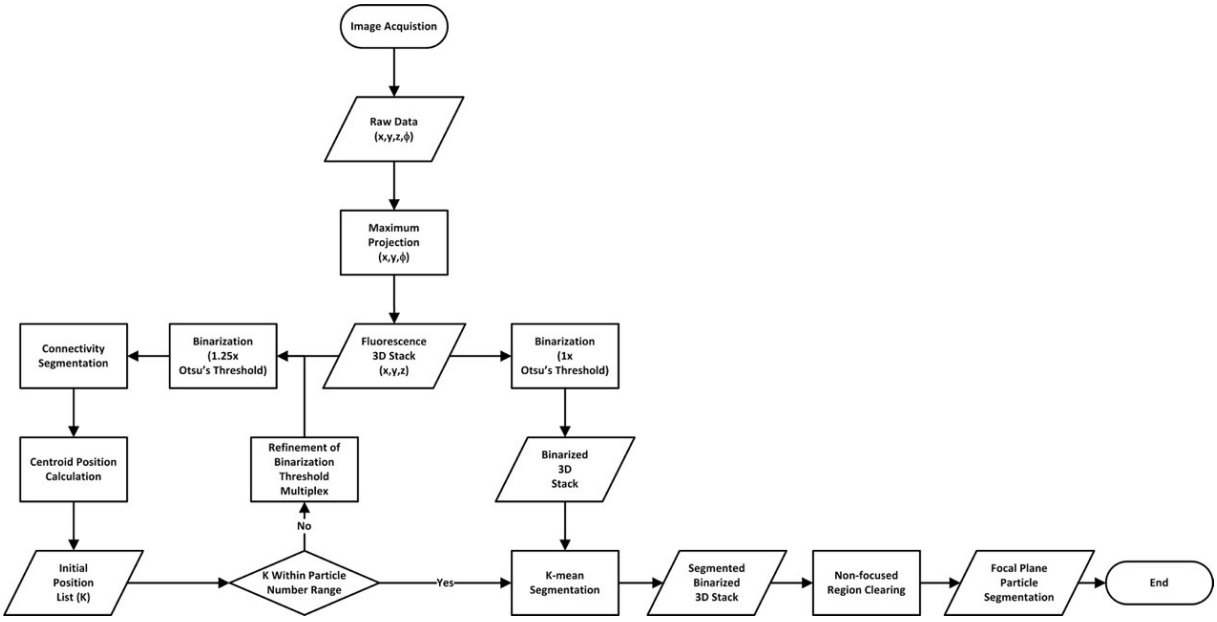

Fig. S1. Flowchart showing the entire process to analyze the 3D positions of the beads within the hydrogel from the acquired image stack.

Oxygen Calibration of CPOx Beads

To calibrate the oxygen concentration of CPOx beads, various gases including: pure nitrogen, and a 50% oxygen-50% nitrogen mixture are first purged into a homemade environmental chamber with dimensions of 127 mm⌀85 mm⌀85 mm (Fig. S2(a)) separately. According to the Stern-Volmer equation,

$$[O_2] = \frac{1}{K_q}\left(\frac{\tau_0}{\tau} - 1\right) \tag{S1}$$

the quenching coefficient K_q can be estimated from the measured τ_0 and τ, which are the fluorescence lifetimes without and with known oxygen concentration, respectively. Here, a 3.5 mm dish filled with 5 mg/ml RTDP (tris(2,2'-Bipyridyl) ruthenium(II) chloride hexahydrate, Acros Organics) solution is used to accurately characterize the oxygen concentration within the chamber[1]. In the experiment, the environmental oxygen concentrations are measured to be 1.09% and 40.95% during the introduction of pure nitrogen and a mixture, respectively. Under these two conditions, the average lifetimes of CPOx beads from three different vials are 8.18 μs and 4.59 μs (Table S1 and Table S2). Therefore, the quenching coefficient can be calculated to be 1.90 from Equation S1. For further validation, we use the averaged lifetime of CPOx beads, 5.84 μs (Table S3), while purging air into the chamber, as τ in Equation S1. The oxygen concentration can then be estimated to be 20.99%, which is close to the normal oxygen concentration in air.

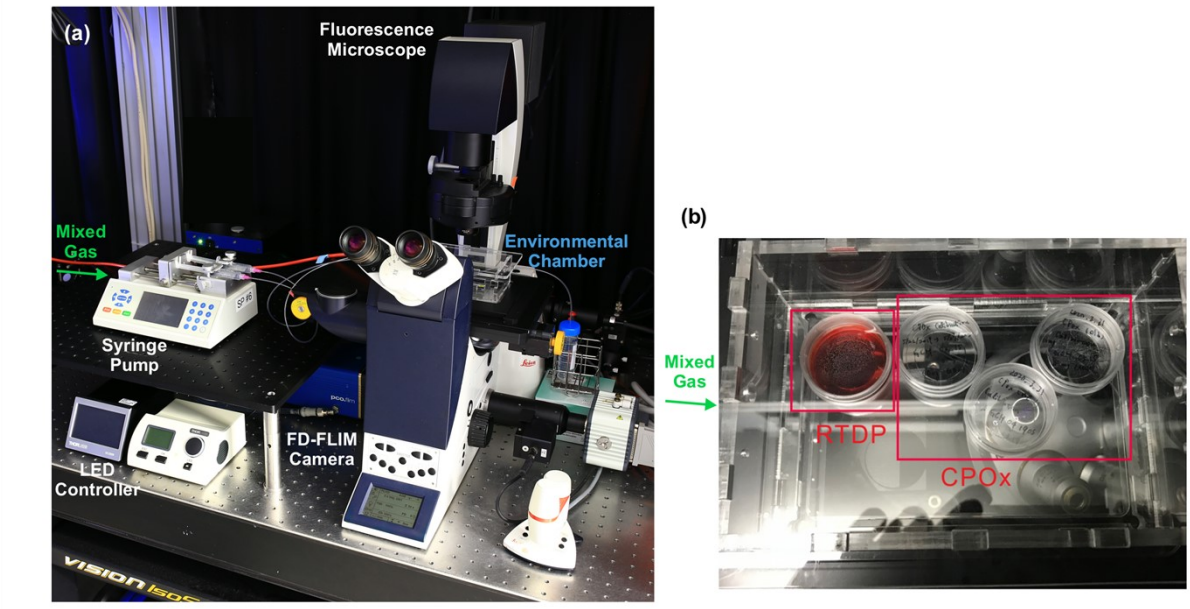

Fig. S2. (a) Photo of the experimental setup for the FD-FLIM system for oxygen calibration of CPOx beads. (b) The RTDP solution placed in a petri dish is exploited to monitor the actual environmental oxygen concentration in the environmental chamber of the device during the experiments. In total, three vials of CPOx beads are measured.

Table S1. The average lifetimes of CPOx beads while introducing pure nitrogen into the chamber.

No. of Vials	Lot Information	Number of Beads	Average Lifetime (μs)
1	GCH 03-15	7	8.09
2	GCH 03-15	16	8.06
3	GCH 04-19OS	12	8.39

Table S2. The average lifetimes of CPOx beads while introducing a 50% oxygen-50% nitrogen mixture into the chamber.

No. of Vials	Lot Information	Number of Beads	Average Lifetime (μs)
1	GCH 03-15	11	4.75
2	GCH 03-15	10	4.58
3	GCH 04-19OS	19	4.51

Table S3. The average lifetimes of CPOx beads while introducing air into the chamber.

No. of Vials	Lot Information	Number of Beads	Average Lifetime (μs)
1	GCH 03-15	12	5.93
2	GCH 03-15	9	5.90
3	GCH 04-19OS	27	5.79

References

S1. H. M. Wu, T. A. Lee, P. L. Ko, W. H. Liao, T. H. Hsieh and Y. C. Tung, *Analyst*, 2019, **144**, 3494-3504.