

Label-free visualization and quantification of fungal-induced calcium carbonate precipitation for self-healing concrete applications

Martí Pla-Ferriol

Lund University

Sophie J.L. van Velzen

Lund University

Calley Eads

Lund University

Edith C. Hammer

Lund University

Dimitrios Floudas

Lund University

Hanbang Zou

hanbang.zou@biol.lu.se

Lund University

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Abstract

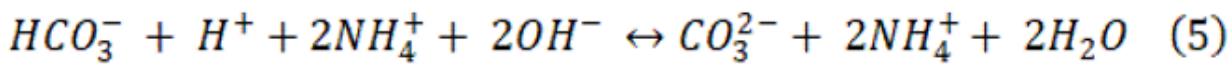
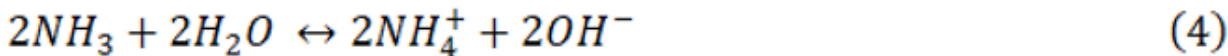
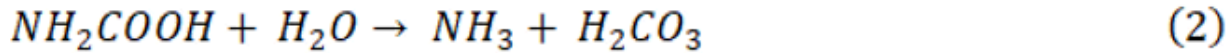
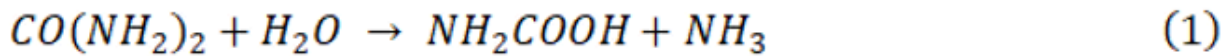
Fungal self-healing concrete, via fungi-induced calcium carbonate precipitation (FICP), is an emerging sustainable solution for infrastructure maintenance. However, the pore-scale mechanisms underlying this process remain poorly understood due to the lack of methods for visualizing and tracking calcium carbonate precipitation within opaque porous media. Here, we found that calcium carbonate precipitated by fungi in a complex growth medium exhibits autofluorescence, whereas calcium carbonate formed in a defined medium does not. We exploited this property as a label-free imaging strategy for real-time visualization and quantification of fungal-induced calcium carbonate precipitation within a microfluidic “crack-on-a-chip” platform. Using this approach, we resolved how different substrate delivery strategies- a single-time addition and periodic additions- regulate the temporal dynamics of biomineralization. Our results showed that urease-mediated CaCO_3 precipitation occurs in bursts synchronized with periodic substrate and nutrient additions. Compared with a single substrate addition, repeated additions significantly increased the number of calcium carbonate crystals, while reducing average crystal size. These findings suggest that a repeated nutrient and substrate supply promotes the formation of new nucleation events rather than enhancing the growth of existing crystals. O-PTIR revealed that the mineral deposits comprised multiple calcium carbonate polymorphs (calcite together with aragonite and/or vaterite). SEM imaging further confirmed extensive spatial overlap between fungal hyphae and calcium carbonate crystals, indicating that fungal biomass and biominerals are intimately associated during precipitation. This study provides a mechanistic basis for controlling biomineralization in self-healing concrete and other biomaterials while advancing the methodology for studying bioprecipitation at the microscale in real time.

Introduction

Concrete is the most utilized artificial building material because of its mechanical properties and low production cost [1]. However, cement production is responsible for approximately 8% of global CO_2 emissions and remains one of the largest contributors to anthropogenic greenhouse gas emissions, in addition to consuming vast quantities of material and posing significant recycling challenges. In Europe, 450–500 million tonnes of construction and demolition waste are generated annually, at least one-third of which is concrete [2]. Extending the service life of concrete infrastructure is therefore recognized as one of the most important strategies for reducing the environmental impact of the construction industry. Among the factors limiting concrete durability, crack formation is particularly critical because it allows the ingress of CO_2 , water, and chloride ions, ultimately leading to corrosion of the embedded steel reinforcement and structural deterioration.

Self-healing concrete through microbially induced calcium carbonate precipitation (MICP) has emerged as a promising strategy for autonomous crack repair. In this process, microorganisms induce the precipitation of calcium carbonate (CaCO_3), which seals cracks and restores water tightness. Several metabolic pathways for MICP exist in nature, including photosynthetic activity in aquatic environments [3], dissimilatory sulphate reduction by sulphate-reducing bacteria [4], denitrification [5] and urea hydrolysis [6]. Among those, urea hydrolysis is less complex, it shows higher CaCO_3 precipitation [7], and it is the most applicable to a building context. In MICP driven by urea hydrolysis, the secreted enzyme urease hydrolyses urea to produce ammonia and carbamic acid (Eq. 1), which is then spontaneously hydrolysed to produce carbonic acid (Eq. 2). These products are then equilibrated in water to form bicarbonate, ammonium and hydroxide ions (Eq. 3–4), increasing

the pH in the medium and resulting in the formation of carbonate ions (Eq. 5). This, ultimately, leads to CaCO₃ precipitation (Eq. 6) in the presence of calcium ions [8, 9].



To date, while research on MICP has focused predominantly on bacteria [10, 11, 12], filamentous fungi are considered a competitive alternative, due to their high surface area that provides abundant nucleation sites for bioprecipitation, their adaptability to harsh dry environments, and their explorative behaviour that allows them to reach complex cracks [13, 14, 15].

Recent studies have demonstrated the feasibility of fungal self-healing concrete. Jin et al. [16] investigated *Aspergillus nidulans* (including genetically modified strains) and other fungi, finding limited growth and calcium carbonate precipitation in *A. nidulans* (MAD1445) and *Trichoderma reesei* (ATCC13631). However, the high pH environment of concrete remains a major challenge for fungal survival and effectiveness. Khushnood et al. [17] embedded *Rhizopus oryzae* and *Trichoderma longibrachiatum* in calcium alginate beads, observing improved compressive strength recovery in cracked cement, particularly with *R. oryzae*. The alginate beads likely modified the environment to support fungal growth. Meanwhile, Zhao et al. [18] used *Neurospora crassa* to form a calcium carbonate coating on mortar, but their nutrient medium, starting at pH 5.5, poses corrosion risks for construction materials. Together, these studies highlight the potential of fungi for self-healing concrete while underscoring the challenges of optimizing fungal performance in harsh, high-pH conditions. Furthermore, some studies have described how different fungal strains can achieve very high efficiency in CaCO₃ precipitation [19, 20].

Despite these advances, our mechanistic understanding of fungal-induced biomineralization remains remarkably limited. Existing studies rely almost exclusively on endpoint characterization techniques such as scanning electron microscope (SEM), X-ray diffraction (XRD), and Scanning Transmission X-ray Microscopy (STXM), providing only a static view of the process after precipitation has occurred.

Consequently, the spatial and temporal evolution of fungal-mediated CaCO_3 mineralization remains poorly understood [21]. In particular, it is still unclear what proportion of a crack or pore is occupied by fungal biomass versus precipitated CaCO_3 , and how these two components evolve during crack healing. Furthermore, the confined pore and crack spaces within concrete contain only limited amounts of nutrients, which may be insufficient to simultaneously sustain fungal growth and support extensive CaCO_3 precipitation. This knowledge gap is critical because the balance between growth and mineral precipitation ultimately determines crack-filling efficiency.

Addressing these questions requires continuous, non-invasive visualization of fungal biomineralization at the pore scale. However, no imaging method currently enables real-time discrimination of fungal biomass and newly precipitated CaCO_3 within opaque porous environments.

Here, we report a label-free imaging strategy that overcomes this limitation. We found that calcium carbonate precipitated by fungi in a complex growth medium exhibits autofluorescence, whereas calcium carbonate formed in a defined growth medium does not. We exploited this unique property as a label-free imaging strategy for real-time visualization and quantification of fungal-induced calcium carbonate precipitation within a microfluidic "crack-on-a-chip" platform.

Using this approach, we visualized and quantified fungal-mediated CaCO_3 precipitation over time under two fungal inoculation methods and two substrate delivery strategies to determine how fungal colonization and substrate availability regulate biomineralization. Our results reveal that periodic substrate replenishment promotes sustained crystal nucleation, while complementary SEM and O-PTIR analyses show the coexistence of multiple calcium carbonate polymorphs and interconnected composites of mycelium and crystals. Together, this work establishes a general platform for investigating fungal biomineralization in real time and provides mechanistic insights that can guide the design of next-generation fungi-based biomaterials.

Materials and methods

Microfluidics

The microfluidic "crack-on-a-chip" device was designed using Computer-Aided Design software (AutoCAD, version 2024.1.5). The device consists of a straight microchannel measuring 20 mm in length, 2.5 mm in width and 100 μm in depth. Within the channel, six rows of cylindrical micropillars (100 μm in diameter) were arranged with a spacing of 300 μm . The 300 μm gap was selected to represent a concrete crack width based on recommendations from Trafikverket (the Swedish Transport Administration), as cracks of this size are considered difficult to detect and repair in practice.

The master mold was fabricated on 4-inch silicon wafers (MicroChemicals GmbH) coated with mr-DWL 100 photoresist (Micro Resist Technology). The microchannel patterns were defined by photolithography using a maskless aligner (MLA150, Heidelberg Instruments). To facilitate demolding of the elastomer, the completed master was coated with an anti-adhesion layer by depositing a self-assembled monolayer (SAM) of perfluorodecyltrichlorosilane (FDTS) using a Fiji atomic layer deposition (ALD) system (Veeco).

Polydimethylsiloxane (PDMS; Sylgard 184, Dow) was prepared according to the manufacturer's instructions, poured over the master mold, and cured to replicate the microchannel structures. After curing, the PDMS replica

was peeled from the master, inlet and outlet ports were punched, and the PDMS slab was bonded to a glass coverslip to form enclosed microfluidic channels. The assembled device was subsequently housed inside a custom-cut Petri dish to maintain sterility and humidity.

Fungal strain and growth media

The fungal strain used in this study is *Clonostachys rosea* (FDO-10), isolated in our lab from an environmental sample. It was maintained regularly in YMG agar plates (malt extract 10g/L, dextrose 4g/L, yeast extract 4g/L, agar 15g/L) adjusted to pH 7. The growth medium used in all experiments was a modified version of Christensen's Urea Medium [22], containing 1g/L peptone, 5g/L NaCl, 1g/L dextrose, 0.2g/L KH₂PO₄, 9g/L urea, and 16.6 g/L CaCl₂, adjusted to pH 7. The defined growth medium used to test the crystal fluorescence was a modified version of Highley medium [23], containing NH₄NO₃ (2g/L) and dextrose (4g/L) as nitrogen and carbon sources, respectively.

Chip inoculation

Two inoculation methods were evaluated to identify conditions that maximize fungal growth and biomineralization within the microfluidic chips as shown in Fig. 1.

For the “SSD-A” treatment (Fig. 1a.1), a culture of the fungus *C. rosea* was used, maintained on YMG medium agar plates. Before inoculation, one side of the microfluidic chip was cut open to provide an entry point for fungal growth. A square agar plug containing actively growing mycelium was excised from the colony margin and placed with the mycelium facing downward and in direct contact with the chip opening. The chip was then filled from the opposite inlet with 5 μ L (the full internal volume of the microchannel) modified Christensen medium (containing no urea or CaCl₂) to promote hyphal growth into the microfluidic channel. After 7 days of incubation, when the fungal hyphae covered most of the channel, the nutrient medium was replaced with 10 μ L modified Christensen medium (containing urea and CaCl₂) and time-lapse imaging was initiated immediately. Excess medium was injected to ensure complete replacement of the previous medium.

For the “Liquid” (SSD-L and PSD-L) method (Fig. 1a.2 and 1b), the same fungal strain was first cultured in liquid medium (modified Christensen medium containing no urea or CaCl₂) for 6 days. The resulting mycelial mat was homogenized using a blender (Omni-mixer 17106, Dupont) at 12000 rpm for 30 seconds to generate a suspension of mycelial fragments. The homogenized suspension was injected into the microfluidic chip and allowed to establish for 24 h, to obtain a more homogeneous distribution of fungal biomass within the chip. Immediately after that, 10 μ L of the modified Christensen medium containing urea and CaCl₂ was introduced into the chip, marking the start of the time-lapse experiment.

Automated substrate delivery

As shown in Fig. 1b), periodic substrate delivery (PSD-L) was performed by delivering modified Christensen medium (with urea and CaCl₂) using a Fluigent Flow EZ pressure controller coupled to an M-SWITCH™ bidirectional microfluidic valve and an M Flow Unit flow sensor (Fluigent) for real-time flow monitoring and closed-loop flow control. The system was programmed using Fluigent All-in-One (A-i-O) software. The flow rate

was maintained at $0.5 \mu\text{L min}^{-1}$ using feedback from the flow sensor. The M-SWITCH™ sequentially connected the pressure controller to each of the four microfluidic chips. Each chip received fresh medium for 10 min (delivering a total of $5 \mu\text{L}$ to fully replace the whole internal volume of the channel), after which the valve automatically switched to the next chip. Following perfusion of all four chips, the system remained idle for 9 h 20 min, resulting in a 10 h interval between consecutive substrate deliveries to the same chip. This automated protocol was repeated throughout the experiment to provide periodic replenishment of nutrients and precipitation substrates while minimizing manual intervention.

Image acquisition

Time-lapse imaging was performed using a Nikon Ti2-E inverted microscope (Nikon Instruments, Japan) equipped with a Nikon Qi2 scientific CMOS camera and controlled using NIS-Elements software. Brightfield and label-free fluorescence images were acquired using a 4× S Fluor objective (working distance, 15.5 mm). Fluorescence from fungal-induced calcium carbonate was detected using a Nikon DAPI filter set (excitation: 356 nm; emission: 477 nm). No fluorescent dyes or labels were used throughout the experiments.

To maximize imaging throughput, four replicate microfluidic chips were bonded onto a single glass coverslip and enclosed within a custom Petri dish. The microscope was programmed to image the four chips sequentially using the automated large-area scan function in NIS-Elements. For each chip, the entire microfluidic channel was defined as a stage area. Adjacent image tiles were acquired with 5% overlap and automatically stitched using image registration to reconstruct the complete channel. To compensate for slight variations in chip flatness, a focus surface was generated for each chip before time-lapse imaging and applied throughout image acquisition to maintain accurate focus across the entire scan area.

Time-lapse imaging was performed for 50 h, with images acquired every 15 min. At each time point, the microscope automatically acquired brightfield and autofluorescence images from all four chips using identical acquisition settings. A z-stack spanning $25 \mu\text{m}$ was acquired at each imaging position with a $12.5 \mu\text{m}$ step size. Brightfield and fluorescence images were captured sequentially at each focal plane. Exposure time, illumination intensity, and camera settings were kept constant throughout the experiment to enable quantitative comparison of autofluorescence intensity over time.

Fluorescent calcium carbonate crystals

Previous studies [24, 25] showed that organic molecules incorporated into calcium carbonate crystals generate lattice strain and structural defects. Fungal hyphae exude a large variety of organic compounds [10] which can potentially be included as impurities into the forming crystals. In addition, one study showed that crystal defects contribute to the luminescence of carbonate minerals [26]. In geological systems, calcite containing occluded organic matter, such as humic acids, has also been reported to exhibit fluorescence [27, 28]. Similarly to these previous findings, we noticed that precipitations in a complex growth medium (modified Christensen's medium) containing peptone extract resulted in fluorescent fungal-derived CaCO_3 crystals. Our previous scanning transmission X-ray microscopy (STXM) analysis [29] showed that similar fluorescent precipitates consisted of a mixture of organic molecules and multiple calcium carbonate polymorphs. These findings suggest that organic components present in the complex medium become incorporated into or occluded within the mineral during biomineralization and are associated with the observed fluorescence.

We therefore tested whether the composition of the growth medium influences the fluorescence of the resulting CaCO_3 crystals. We compared urease-mediated calcium carbonate precipitation (Fig. 2a) in a defined medium (modified Highley medium) and in a complex medium (modified Christensen medium) containing high-molecular-weight organic components (peptone extract). We found that CaCO_3 precipitated by the fungal strain in a complex medium exhibited strong autofluorescence detectable in the DAPI channel (Fig. 2a3, 2b, 2c), whereas crystals formed in a defined medium showed no fluorescence. While systems using a defined growth medium still could produce CaCO_3 crystals when supplied with pure urease enzymes, the forming crystals lacked any fluorescence. Using a complex medium resulted in fluorescent crystals but those were smaller and fluorescence was profoundly lower, compared to the crystals produced in the vicinity of the fungal hyphae. However, the exact underlying mechanism is yet to be explored.

Fluorescence validation

To evaluate the robustness of fluorescence-based crystal size measurements, DAPI-segmented crystals were compared with manually annotated brightfield (BF) images, which served as the reference standard. Each crystal was analyzed individually by matching the DAPI-derived binary mask to the corresponding BF annotation. Segmentation performance was quantified using the Intersection over Union (IoU) and Dice similarity coefficient, while measurement accuracy was evaluated using relative area error and equivalent diameter agreement. Intersection over Union (IoU) measures how much the DAPI segmentation overlaps with the manually annotated BF mask. It is calculated as the overlapping area divided by the total area covered by either mask. The Dice coefficient was calculated as twice the area of overlap between the DAPI segmentation and the manually annotated BF mask divided by the sum of the areas of the two masks.

A total of 27 crystal instances from six representative cropped images spanning different experimental treatments were manually annotated and used for validation. All 27 crystals were successfully detected and matched between the BF annotations and the DAPI segmentations. The DAPI-based segmentation achieved a mean IoU of 0.725 and a mean Dice coefficient of 0.825. The mean relative area error was 10.7%, while the mean equivalent diameter error was only 3.2%, indicating that although small differences in mask boundaries were present, perhaps due to manual annotation error, fluorescence-based segmentation provided highly accurate estimates of crystal size.

Image pre-processing

Raw TIFF image sequences (DAPI) were processed using a custom MATLAB script [30]. For each sequence, a region of interest (ROI) was manually defined using a polygon mask to exclude image borders and artifacts. Background illumination was estimated for each frame using morphological opening with a disk-shaped structuring element (radius = 20 pixels) and subtracted from the corresponding frame to correct for uneven illumination. Temporal subtraction was then applied by subtracting the illumination-corrected first frame from each subsequent frame, isolating newly appearing structures from the background. Binary masks were generated by thresholding at the 99.5th percentile of pixel intensities within the ROI. Binary images were saved as 8-bit TIFF files for compatibility with downstream tracking software.

Particle Tracking

Crystal detection and tracking were performed using the plugin “TrackMate” v7.14.0 in Fiji [31, 32]. The Mask Detector setting was used to detect fluorescent crystals directly from binary images, with an initial quality threshold applied to remove low-confidence detections (quality > 50). Detected crystals were further filtered by a minimum radius threshold (radius > 8.1 μm) to exclude noise. Tracking was performed using the “LAP Sparse Tracker” with a maximum linking distance of 15 μm between consecutive frames. Gap closing was allowed over a maximum of 2 frames with a maximum gap closing distance of 15 μm . Track splitting and merging were disabled. To ensure no other particles aside from the fluorescent crystals were included, tracks were filtered to retain only those starting after frame 2 and ending after frame 199 out of 200 total frames. The same detection and tracking parameters were applied consistently across all treatments and replicates.

Data Analysis

All downstream analyses were performed in R (version 4.4) [33]. Spots with circularity below 0.25 were excluded as noise prior to analysis.

Crystal count and nucleation rate were tracked over time for each replicate. To characterize growth dynamics, mathematical models were fitted to the mean crystal count per timepoint. For “SSD-A” and “SSD-L” treatments, which showed rapid initial nucleation followed by a plateau, an exponential rise to plateau model was applied: $y = \text{Asym} \times (1 - e^{(-k \times t)})$. For the “PSD-L” treatment, which showed continuous linear growth, a linear model was used. Model goodness of fit was assessed using the coefficient of determination (R^2).

Mean crystal area over time was modelled using a logarithmic fit ($y = a + b \times \log(t)$) for all three treatments, which provided the best description of the gradual growth followed by a slowdown observed across all treatments (SSD-A $R^2 = 0.932$, SSD-L $R^2 = 0.808$, PSD-L $R^2 = 0.929$).

Nucleation rate was defined as the number of new crystals appearing per 15-minute timepoint, calculated as the difference in crystal count between consecutive timepoints. Negative values, arising from crystal merging and other artifacts in the tracking software, were set to zero. The median nucleation rate across replicates was calculated per timepoint and smoothed using locally estimated scatterplot smoothing (LOESS, span = 0.3).

Statistical Analysis

Prior to analysis, data were tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. For crystal count at the final timepoint (50h), Levene's test indicated unequal variances across treatment groups ($p = 0.042$), so a Welch's one-way ANOVA was applied, with pairwise comparisons conducted using the Games-Howell post-hoc test. For crystal size at the final timepoint, individual crystal areas were analysed using a linear mixed effects model with treatment as a fixed effect and biological replicate as a random effect, fitted using the *nlme* package [34]. in R, to properly account for the nested structure of the data (individual crystals nested within replicates). Statistical significance was defined as $p < 0.05$.

Scanning electron microscopy (SEM) analysis

To produce samples for SEM, the fungi were inoculated in the microfluidic device using the agar plug inoculation method. The channels were filled with 20 μL modified Christensen medium (containing no urea or CaCl_2). A small agar plug from a stock culture plate of fungi was placed upside down in the reservoir to inoculate the device. After 7 days of incubation, 20 μL Christensen medium containing 150 mM CaCl_2 and urea was added to the reservoir. Following a further 7 days of incubation, an additional 20 μL Christensen medium containing 150

mM CaCl_2 and urea was added, after which the chips were incubated for another 7 days. The PDMS layer was then carefully removed from the glass slide before being air-dried.

The air-dried samples were mounted onto SEM stubs using double-sided carbon tape before being sputter-coated with gold (Cressington 108 Auto, 20 mA, 85 s). Imaging was performed using a Hitachi SU3500 scanning electron microscope operated at 5 kV with a secondary electron detector.

O-PTIR

Single-point optical photothermal infrared (O-PTIR) spectra were acquired using a mIRage microscope (Photothermal Spectroscopy Corp., Santa Barbara, CA, USA) equipped with a four-chip pulsed quantum cascade laser (QCL) system (Daylight Solutions, San Diego, CA, USA). The QCL operated at a pulse rate of 100 kHz and covered a tunable mid-IR spectral range of 770–1802 cm^{-1} . Spectra were collected at a spectral resolution of 6.6 cm^{-1} using 8 averaged acquisitions under dry-air purge to minimize atmospheric water absorption.

Brightfield optical images were first collected using a 10x refractive objective to identify crystals within the exposed microfluidic channels. O-PTIR spectra were then acquired in co-propagating reflection mode using a 40x Cassegrain objective (0.78 NA). A colinear 532 nm probe laser was used to detect the photothermal response, providing a diffraction-limited spatial resolution of ~ 500 nm throughout the mid-IR range.

Approximately eight point spectra were acquired from different spots on each crystal. Autofocus was performed prior to each measurement to optimize O-PTIR signal and ensure a similar working distance across all spots. All spectra were collected using PTIR Studio 4.6 (Photothermal Spectroscopy Corp.). Spectra exhibiting substantial overlap from the surrounding fungal hyphae were excluded from further analysis. Consequently, between two and six representative spectra per crystal were retained for analysis, depending on the extent of fungal spectral contributions. Spectra were intrinsically background corrected, and instrumental step discontinuities arising from stitching of the four QCL modules were corrected within PTIR Studio using the built-in step correction (1454 cm^{-1} , window size = 5).

Spectral preprocessing was performed in Quasar Spectroscopy software and consisted of spectral truncation to 1755 – 960 cm^{-1} , linear baseline correction, Savitzky-Golay smoothing (window size = 5, second-order polynomial), vector normalization, and k-means clustering ($k = 2$) to identify groups of similar spectra.

Results and discussion

We exploited the autofluorescence of fungal-induced calcium carbonate crystals as a label-free imaging strategy to visualize and quantify time resolved fungal-induced calcium carbonate precipitation process. Two substrate delivery strategies and inoculation methods were compared. Periodic substrate addition significantly increased the amount of crystal numbers and thus covered more pore space than a single-time addition, but had little effect on crystal size. The nucleation rate exhibited a consistent time-lagged response to periodic substrate inputs. SEM and O-PTIR imaging further revealed an interconnected three-dimensional mycelial scaffold surrounding the mineral deposits.

Repeated nutrient and substrate supply promotes CaCO_3 biomineralization

Crystal count over time followed distinct patterns depending on the treatment. SSD-A and SSD-L treatments showed rapid initial nucleation followed by a plateau (Fig. 2A), well described by an exponential rise to plateau model (SSD-A: $R^2 = 0.918$, SSD-L: $R^2 = 0.923$). In contrast, the periodic nutrient supply, “PSD-L”, treatment showed continuous linear growth throughout the 50-hour experiment ($R^2 = 0.975$). This suggests that a periodic addition of nutrients and substrates allows for an increase in the number of precipitated crystals by producing new nucleation events over time, in contrast to the single burst of nucleation events observed in the single nutrient supply (SSD-A and SSD-L) treatments.

Analysis of the nucleation rate confirmed the differences in growth dynamics between treatments (Fig. 2B). The SSD-L treatment showed a sharp peak in nucleation rate at the start of the experiment, rapidly declining to near zero by 10 hours. The SSD-A treatment showed a similar but less pronounced early peak, with a gradual decline over the first 20 hours. The PSD-L treatment showed a distinct pattern, with nucleation rate peaks recurring at approximately 10-hour intervals, synchronized with the timepoints of nutrient and substrate addition ($T = 0, 10, 20, 30$, and 40 hours), suggesting that each nutrient and substrate pulse triggered a new wave of crystal nucleation.

Crystal count (Fig. 2C) at the final timepoint (50h) differed significantly between treatments (Welch's one-way ANOVA, $F = 14.35$, $p = 0.013$). Post-hoc analysis revealed that the PSD-L treatment had significantly more crystals than both the SSD-A (Games-Howell, $p = 0.021$) and SSD-L (Games-Howell, $p = 0.015$) treatments allowing the formation of significantly more crystals than the single delivery treatments. No significant difference in crystal count was found between the SSD-A and SSD-L treatments (Games-Howell, $p = 0.884$).

Crystal area increased over time in all three treatments following a logarithmic trend, indicating rapid initial growth that gradually slowed over time (Fig. 4A). The logarithmic model provided a good fit for the PSD-L treatment ($R^2 = 0.929$) and SSD-A ($R^2 = 0.932$), while the fit for SSD-L was more modest ($R^2 = 0.808$).

Analysis of individual crystal areas at 50h (Fig. 3C) using a linear mixed effects model revealed significant differences in crystal area between treatments ($F = 90.49$, $p < 0.0001$). Both SSD-A and SSD-L treatment crystals had significantly larger areas than PSD-L crystals ($p < 0.0001$ for both comparisons), while no significant difference in crystal area was found between SSD-A and SSD-L treatments ($p = 0.261$). Combined with the significantly higher crystal count in the PSD-L treatment, these results suggest that repeated nutrient pulses promote continuous nucleation of new small crystals rather than growth of existing ones, resulting in a larger number of smaller crystals compared to the SSD-A and SSD-L treatments.

OPTIR and SEM results show a combination of organic material and CaCO_3 crystals

Figure 5. SEM pictures of calcium carbonate crystals precipitated by the same fungus in the same chip. a-c) fused crystals mixed with fungal mycelium at different scale d) rhombohedral crystal with rough surface with a fungal hypha through it.

After carefully peeling the PDMS slab from the glass coverslip, the fungal mycelium and mineral deposits remained intact on the coverslip, enabling unprecedented visualization of their spatial relationship by scanning electron microscopy. As shown in Fig. 5, a dense mycelial network extended throughout the surface, illustrating

how the fungus explored and colonized the available space within the microfluidic channel. The mycelial network formed an interconnected three-dimensional scaffold around the mineral deposits. In many cases, hyphae were observed in direct contact with the crystal surfaces, with individual hyphae extending across or appearing partially embedded within the mineral aggregates, indicating intimate interactions between the fungal biomass and the precipitated minerals. The mineral deposits consisted of irregular, faceted crystal aggregates with rough, textured surfaces. While some crystals exhibited faceted morphologies resembling rhombohedral calcite, others displayed more irregular morphologies, suggesting the coexistence of multiple calcium carbonate polymorphs which is consistent with the result from our previous work [24]. In addition, several mineral aggregates appeared to consist of multiple fused crystallites, indicating continued crystal nucleation and growth on the fungal network.

To verify the chemical composition of these mineral deposits, optical photothermal infrared spectroscopy (O-PTIR) was performed. By combining infrared spectroscopy with sub-micrometre spatial resolution (~ 500 nm), O-PTIR enables direct chemical analysis of individual crystals while preserving their spatial association with fungal hyphae.

O-PTIR point spectra were collected from six representative crystals (Fig. 6a-f) with multiple measurements (2–6) acquired for each crystal (Fig. 6g). K-means clustering of the spectra identified two groups based primarily on differences in the relative intensities of the carbonate bands at 1486, 1410, and 1074 cm^{-1} (Fig. 6h), indicating subtle chemical variations among the crystals. All spectra exhibited characteristic carbonate absorptions at approximately 1486, 1410, and 1074 cm^{-1} , corresponding to the ν_3 asymmetric and ν_1 symmetric stretching modes of the CO_3^{2-} group. These spectral features confirm that the mineral deposits associated with the fungal network are composed of calcium carbonate, providing direct evidence of fungal-induced calcium carbonate precipitation. The coexistence of the ν_3 bands at 1410 and 1486 cm^{-1} together with the IR-active ν_1 band at 1074 cm^{-1} is consistent with a mixture of calcium carbonate polymorphs, including calcite together with aragonite or vaterite. However, the overlapping infrared signatures of aragonite and vaterite bands do not allow for unambiguous differentiation using O-PTIR alone.

Occasionally, spectra also contained contributions from fungal hyphae, including amide I (~ 1660 cm^{-1}), amide II (1550 cm^{-1}), and polysaccharide bands between 1000 and 1200 cm^{-1} . Spectra selected for analysis were chosen to minimize these contributions, although a small residual fungal signal remains in some measurements.

In conclusion, we found that fungal-induced calcium carbonate precipitated in a complex growth medium exhibits autofluorescence that allows the development of a label-free imaging strategy for real-time visualization of fungal biomineralization. By integrating this autofluorescence with a microfluidic platform, we established a quantitative imaging system capable of continuously monitoring fungal growth, crystal nucleation, and calcium carbonate precipitation at the pore scale. This approach overcomes a major limitation of existing endpoint characterization techniques by capturing the dynamic evolution of biomineralization as it occurs.

Using this platform, we demonstrated that nutrient delivery plays a critical role in regulating fungal-induced calcium carbonate precipitation. Periodic substrate replenishment promoted repeated nucleation events and significantly increased the number of precipitated crystals compared with conventional single-dose treatments. Furthermore, SEM and O-PTIR analyses revealed that fungal hyphae and calcium carbonate form an

interconnected three-dimensional composite, providing direct evidence that fungal networks serve as structural scaffolds for progressive crystal nucleation and growth.

More importantly, the label-free imaging strategy shown here is not limited to fungal self-healing concrete. Because it enables non-invasive, real-time visualization of fungal biomineralization without fluorescent labels or destructive sampling, it provides a versatile experimental platform for studying fungal–mineral interactions in a wide range of natural and engineered porous systems.

Abbreviations

FICP

Fungal-Induced Calcium Carbonate Precipitation

MICP

Microbially-Induced Calcium Carbonate Precipitation

SSD-A

Single Substrate Delivery (Agar)

SSD-L

Single Substrate Delivery (Liquid)

PSD-L

Periodic Substrate Delivery (Liquid)

Declarations

Author Contribution

H.Z. formulated the overall project goal. H.Z. and M.P.F. developed the research aims and experimental design. H.Z. designed and fabricated the microfluidic masters, conducted fluorescence evaluation of crystals, and performed image preprocessing. H.Z. was responsible for project administration, produced Figures 1 and 2b–c, and formatted all figures. M.P.F. screened and maintained fungal cultures, developed substrate protocols, validated and replicated experiments, performed data analysis, image analysis and statistical analysis, and produced Figures 2a, 3, 4, and 5. S.J.L.v.V. developed protocols and conducted microfluidic experiments, programmed the automated substrate delivery system, and prepared samples for SEM and O-PTIR analyses. C.E. conducted O-PTIR experiments, performed data analysis, contributed to the interpretation and discussion of the O-PTIR results, and produced Figure 6. H.Z. and E.C.H. provided resources for the experiments. E.C.H. and D.F. provided guidance on experimental design and contributed to the interpretation and discussion of the results. H.Z. and M.P.F. curated the data for publication. H.Z., C.E., and M.P.F. wrote and edited the original draft. H.Z., D.F., and E.C.H. contributed to funding acquisition.

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Data Availability

The data generated and analyzed during this study are available from the corresponding author upon reasonable request due to the large file sizes.

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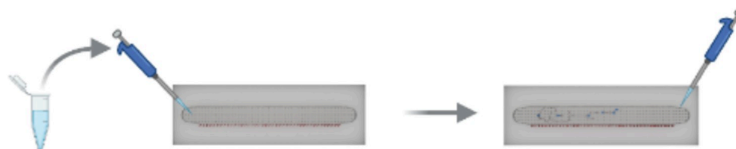
Figures

a) Single Substrate Delivery

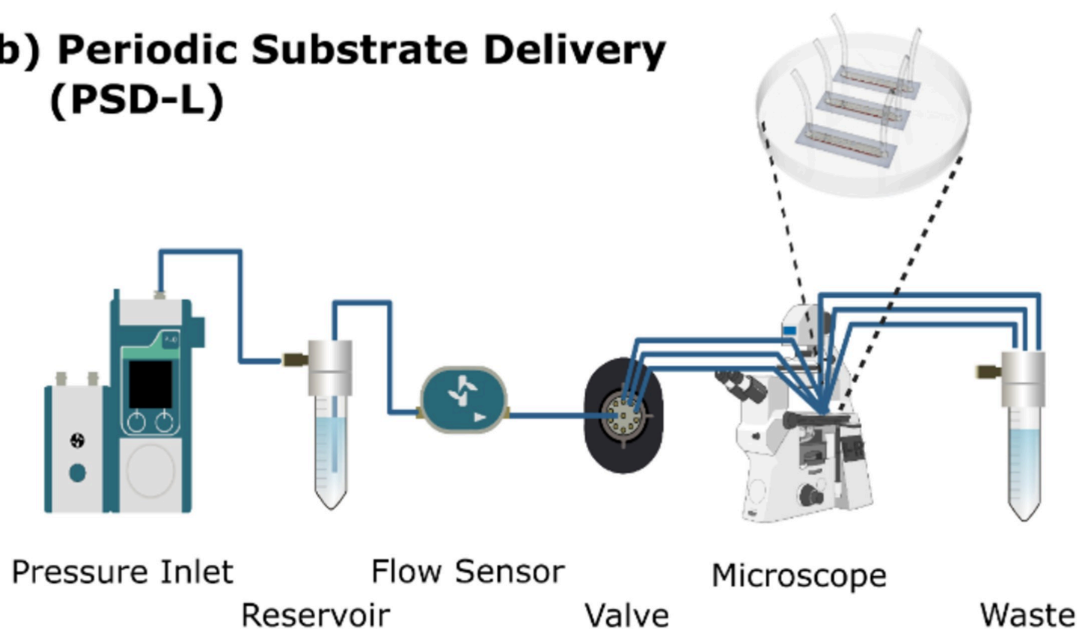
1) Agar (SSD-A)



2) Liquid (SSD-L)



b) Periodic Substrate Delivery (PSD-L)



c) Spatiotemporal Mapping of Crystal Growth

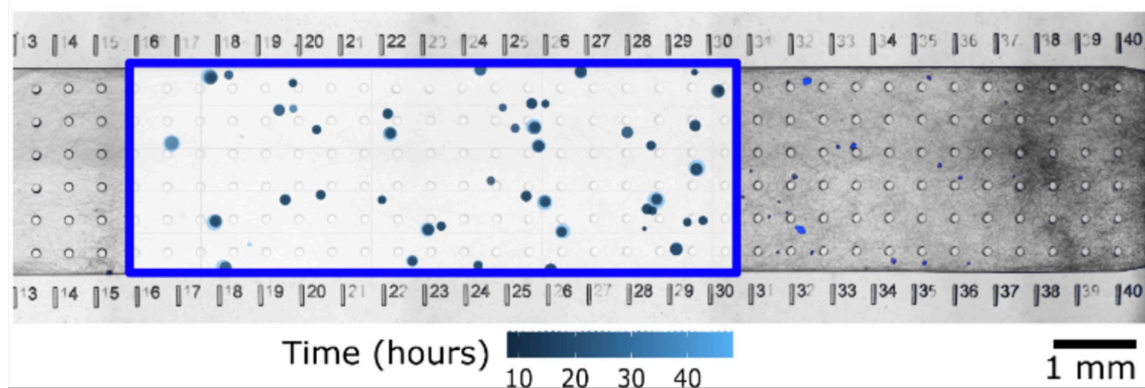


Figure 1

Schematic illustration of experimental setup, depicting different inoculation types and substrate delivery modes for monitoring fungi-induced calcium carbonate precipitation. (a1) Agar inoculation with single substrate and nutrient delivery (SSD-A). (a2) Blended mycelium in liquid inoculation with single substrate and nutrient delivery (SSD-L). (b) Blended mycelium in liquid inoculation with periodic substrate and nutrient delivery using a pump

setup (PSD-L). (c) Illustration of time-resolved image analysis of crystal growth based on fluorescence DAPI signal.

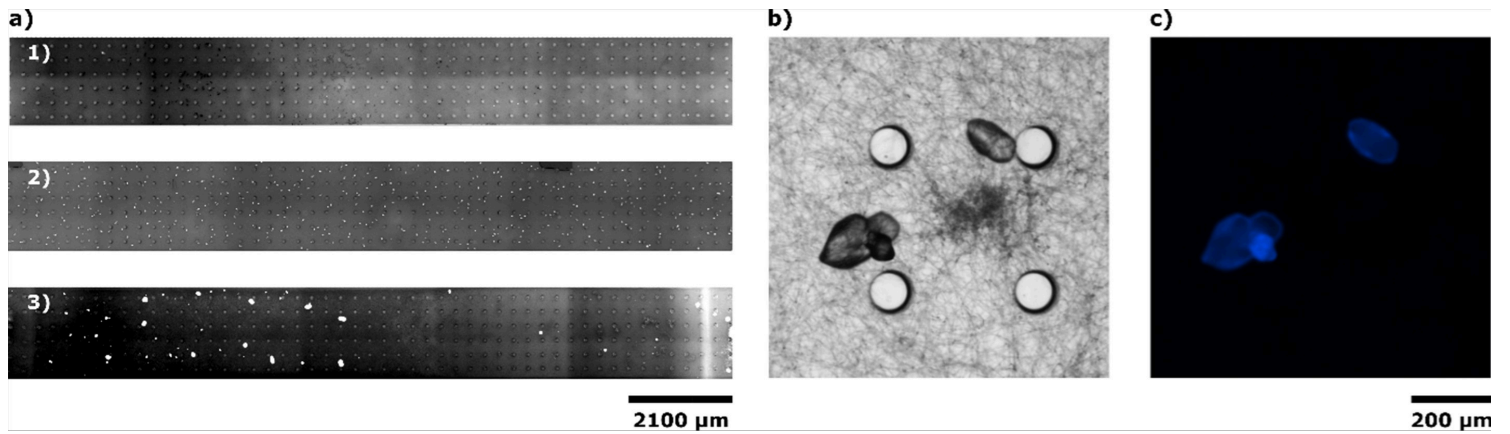


Figure 2

(a1–a3) Comparison tests to determine whether the presence of the fungus or organic molecules in the medium contributes to the fluorescence of the formed crystals. Images were acquired using a DAPI filter. Image brightness was adjusted to facilitate visualization of the microfluidic channel background and non-fluorescent crystals. (a1) Negative control (no fungus): Defined (Highley) growth medium supplemented with urease, CaCl₂, and urea. CaCO₃ crystals formed without detectable fluorescence. (a2) Complex medium control (no fungus): Complex growth medium (modified Christensen medium) supplemented with urease, CaCl₂, and urea. CaCO₃ crystals formed and exhibited fluorescence. (a3) Fungal treatment: Modified Christensen growth medium inoculated with the fungus and supplemented with CaCl₂ and urea. Large CaCO₃ crystal aggregates formed and exhibited strong fluorescence. (b-c) Zoomed-in brightfield (b) and DAPI fluorescence (c) images of CaCO₃ crystals formed by the fungus in modified Christensen growth medium supplemented with CaCl₂ and urea. Fungal hyphae and PDMS microfluidic pillars (100 μm in diameter) are visible in the background.

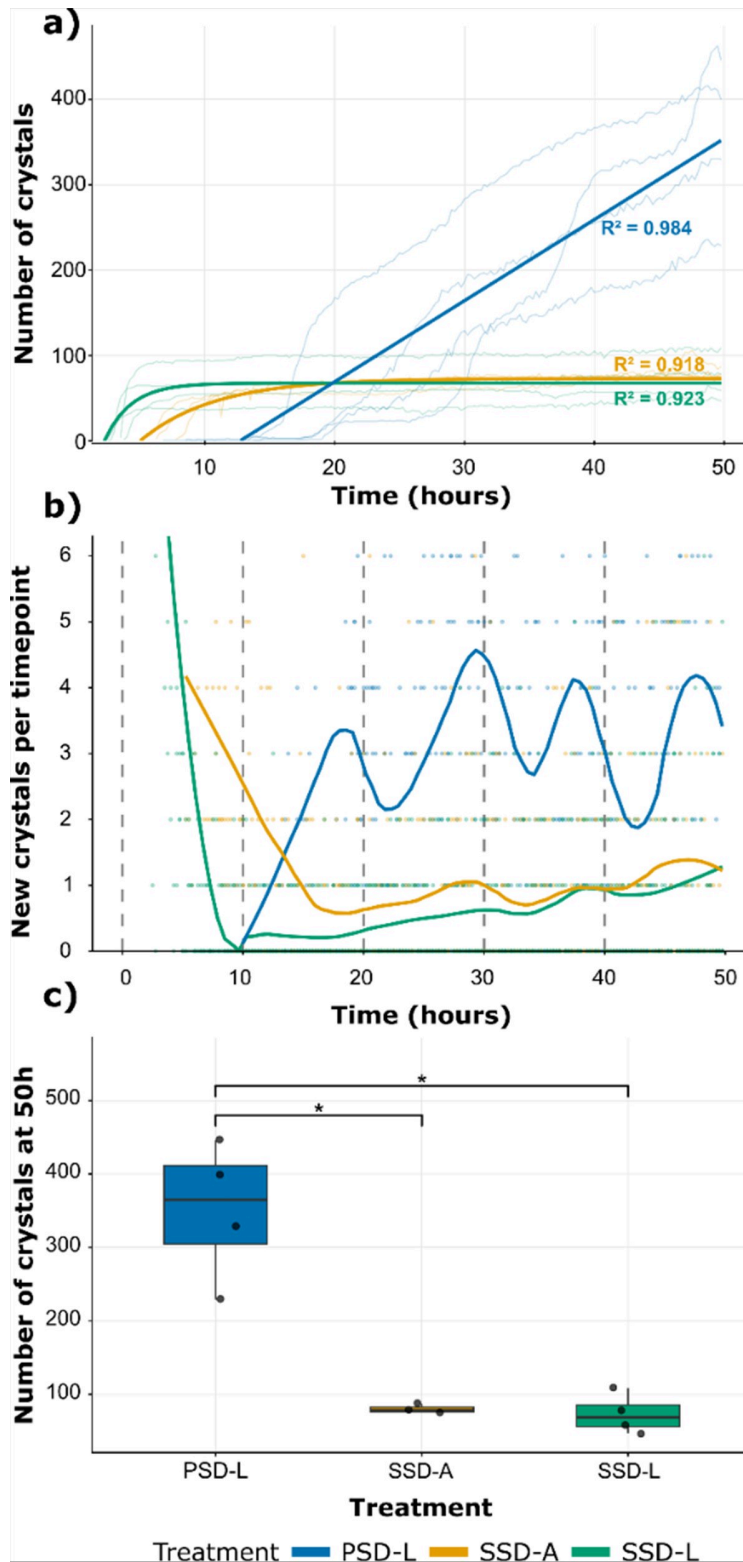


Figure 3

Time-resolved analysis of crystal number at 15-min intervals over a 50-h period for periodic substrate delivery with liquid inoculum (PSD-L, blue), single substrate delivery with agar inoculation (SSD-A, yellow), and single substrate delivery with liquid inoculum (SSD-L, green). (a) Number of crystals detected per treatment over 50 hours. Faint lines represent individual biological replicates ($n = 4$ per treatment for PSD-L and SSD-L, $n = 3$ for SSD-A). Bold lines represent fitted models: exponential rise to plateau for SSD-A ($R^2 = 0.918$) and SSD-L ($R^2 =$

0.923), and linear growth for PSD-L ($R^2 = 0.984$). (b) Crystal nucleation rate over time. Median number of new crystals appearing per 15-minute timepoint per treatment, calculated as the difference in crystal count between consecutive timepoints. Points represent individual replicate measurements per timepoint. Bold lines represent LOESS smoothing (span = 0.3) applied to reveal the underlying trend. Dashed vertical lines indicate timepoints of substrate addition to the PSD-L treatment (T = 0, 10, 20, 30 and 40 hours). (c) Total number of crystals detected per biological replicate at 50 hours, with individual replicate values shown as points overlaid on the boxplot. Significance brackets indicate results from a Welch's one-way ANOVA with Games-Howell post-hoc test. * $p < 0.05$.

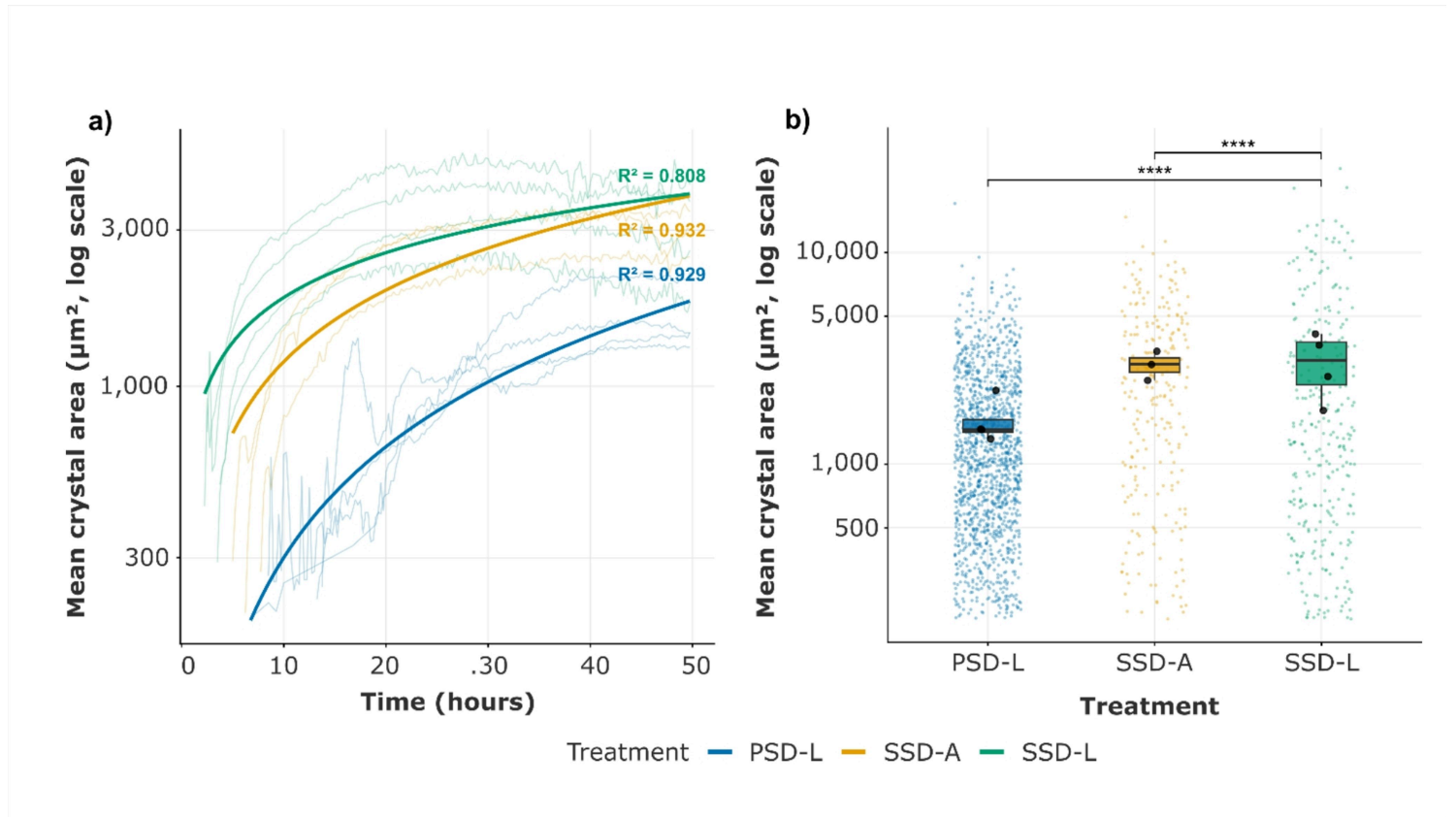


Figure 4

Time-resolved analysis of crystal area at 15-min intervals over a 50-h period for periodic substrate delivery with liquid inoculum (PSD-L, blue), single substrate delivery with agar inoculation (SSD-A, yellow), and single substrate delivery with liquid inoculum (SSD-L, green). (A) Mean crystal area over time. Faint lines represent individual biological replicates ($n = 4$ per treatment for PSD-L and SSD-L, $n = 3$ for SSD-A). Bold lines represent logarithmic fits: SSD-A ($R^2 = 0.932$), SSD-L ($R^2 = 0.808$) and PSD-L ($R^2 = 0.929$). (B) Mean crystal area (μm^2) per biological replicate at 50 hours, with individual crystal measurements shown in the background. Significance brackets indicate results from a linear mixed effects model with treatment as a fixed effect and biological replicate as a random effect. **** $p < 0.0001$

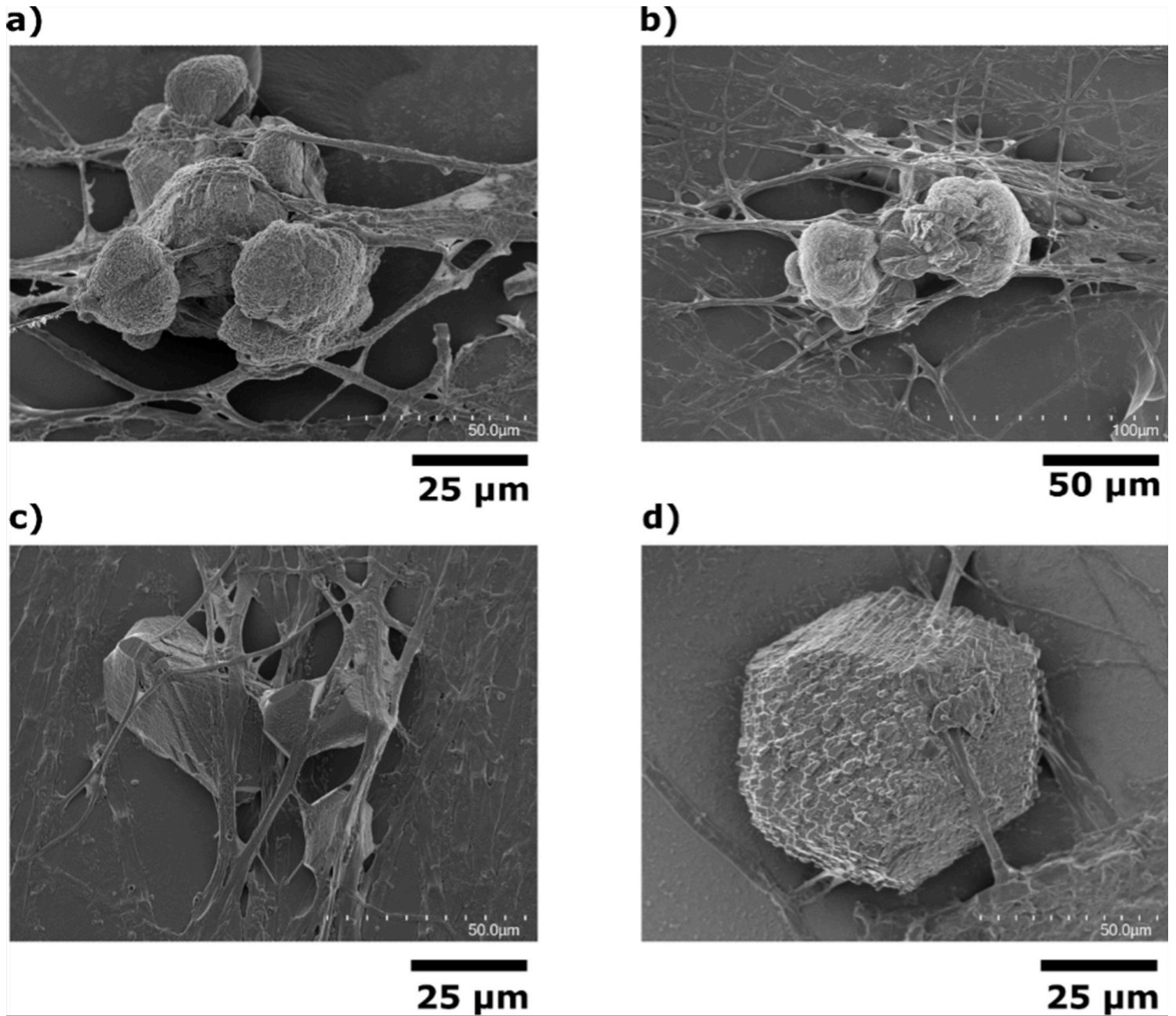


Figure 5

SEM pictures of calcium carbonate crystals precipitated by the same fungus in the same chip. a-c) fused crystals mixed with fungal mycelium at different scale d) rhombohedral crystal with rough surface with a fungal hypha through it.

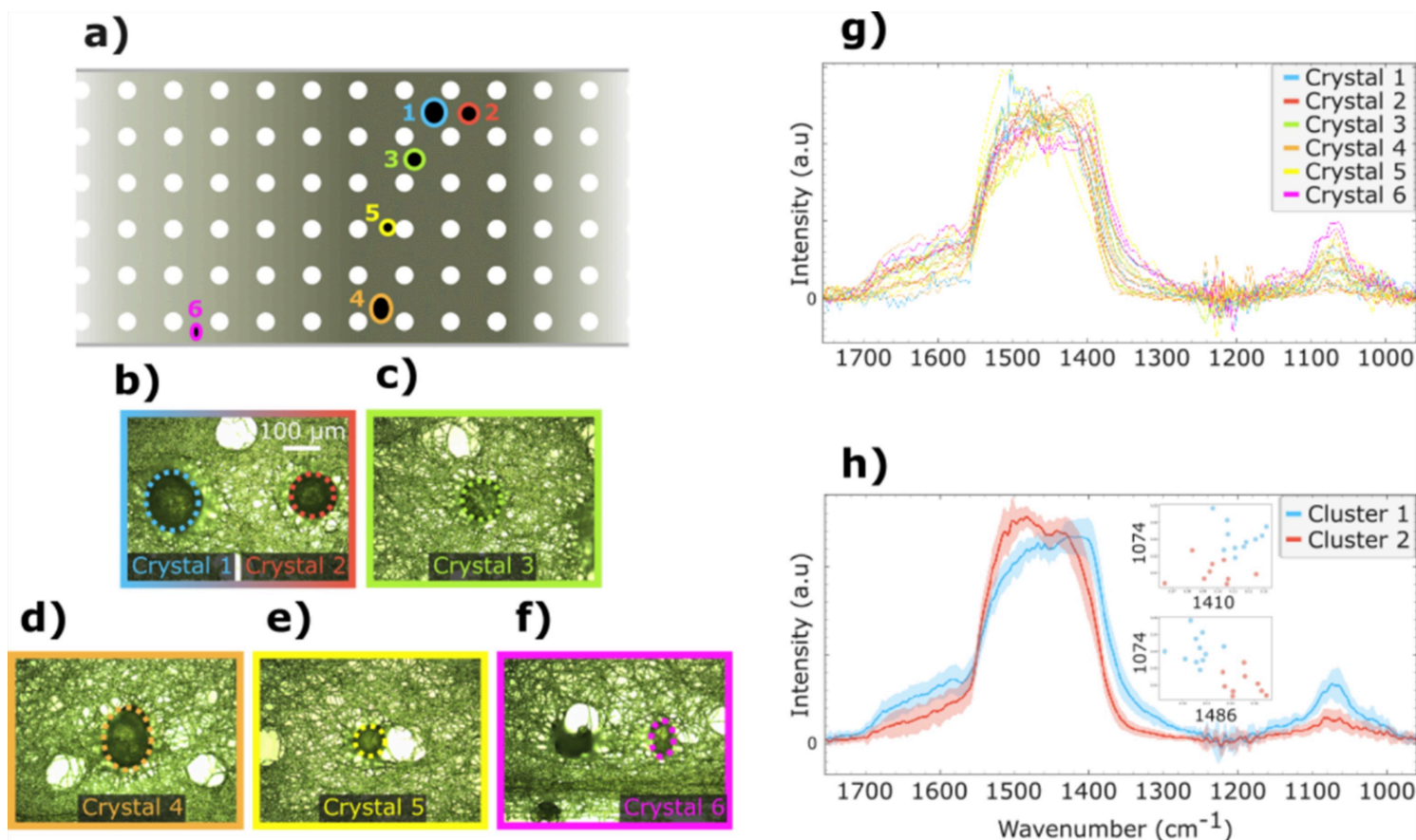


Figure 6

O-PTIR characterization of calcium carbonate crystals in a fungal network. (a) Schematic showing the locations of the measured crystals within the exposed microfluidic chip. (b-f) Optical images of the six analyzed crystals. (g) Individual O-PTIR point spectra acquired from each crystal. (h) Averaged O-PTIR spectra obtained after k-means clustering, with shaded regions indicating the standard deviation. Insets compare the relative intensities of the 1074 cm^{-1} band with the 1410 and 1486 cm^{-1} carbonate bands used for clustering.