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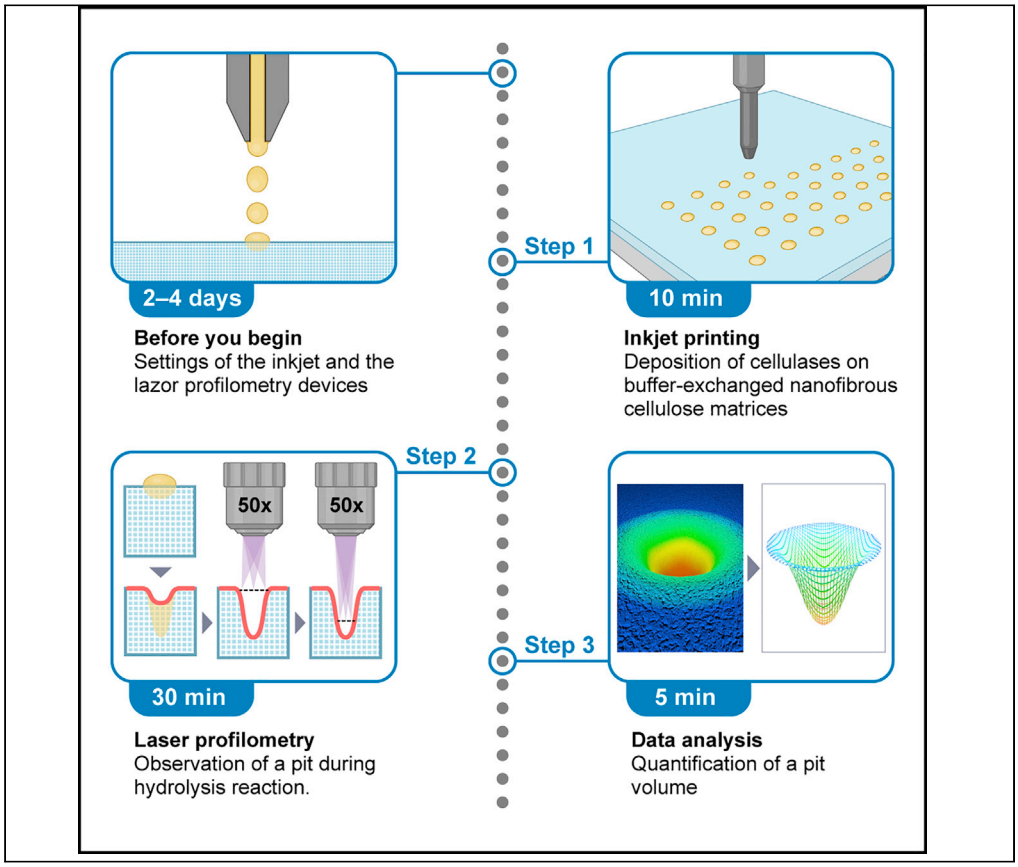
Protocol for analyzing enzymatic hydrolysis of cellulose using surface pitting observation technology



Assaying enzymatic degradation of water-insoluble substrates like cellulose is challenging because only the substrate surface is accessible to the enzymes resulting in low reaction rates. Here, we describe a protocol for surface pitting observation technology (SPOT), an ultra-sensitive quantitative assay for analyzing enzymatic hydrolysis of cellulose. We describe the use of a porous substrate to accelerate the hydrolysis rate of cellulose. We also detail the steps for combining inkjet patterning and optical profilometry to analyze volume loss upon hydrolysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Mikako Tachioka, Mikiko Tsudome, Shigeru Deguchi

tachiokam@jamstec.go.jp (M.T.)
shigeru.deguchi@jamstec.go.jp (S.D.)

Highlights

Nanofiber-based technique for quantifying enzymatic hydrolysis of cellulose at nanoscale

Quantification of volume loss of cellulose matrices using a laser scanning microscope

Inkjet printing technology can deposit a few hundred picograms of cellulases

Can be used to quantify enzymatic hydrolysis of less than 1 ng of crystalline cellulose

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Protocol for analyzing enzymatic hydrolysis of cellulose using surface pitting observation technology

Mikako Tachioka,[1,2,*] Mikiko Tsudome,[1] and Shigeru Deguchi[1,3,*]

[1]Research Center for Bioscience and Nanoscience, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

[2]Technical contact

[3]Lead contact

*Correspondence: tachiokam@jamstec.go.jp (M.T.), shigeru.deguchi@jamstec.go.jp (S.D.)
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SUMMARY

Assaying enzymatic degradation of water-insoluble substrates like cellulose is challenging because only the substrate surface is accessible to the enzymes resulting in low reaction rates. Here, we describe a protocol for surface pitting observation technology (SPOT), an ultra-sensitive quantitative assay for analyzing enzymatic hydrolysis of cellulose. We describe the use of a porous substrate to accelerate the hydrolysis rate of cellulose. We also detail the steps for combining inkjet patterning and optical profilometry to analyze volume loss upon hydrolysis.

For complete details on the use and execution of this protocol, please refer to Tsudome et al. (2022).[1]

BEFORE YOU BEGIN

The protocol below describes a method for measuring the activity of cellulases on nanofibrous cellulose.[1] The quantitative assay of enzymatic hydrolysis of cellulose has usually been insensitive and time-consuming, primarily owing to the recalcitrance and water-insolubility of crystalline cellulose. This newly developed protocol is the first to successfully quantify the nanoscale enzymatic hydrolysis of cellulose. While existing methods for quantifying cellulase activity measure the amount of end-product accumulation,[2–4] this study achieves ultra-sensitive quantification by measuring the volume loss of nanofibrous cellulose. In this protocol, several hundred picograms of cellulases are deposited on a nanofibrous cellulose gel by a piezo-driven inkjet patterning device. Then, the amount of hydrolyzed cellulose is quantified by analyzing the 3D geometry of a pit formed on the surface of nanofibrous cellulose gel using a laser scanning confocal microscope. This protocol can also be used to assay the microbial hydrolysis of nanofibrous cellulose.[1]

> ***Note:*** This protocol was optimized for commercially available cellulase (Meicelase, Meiji Seika) at a concentration of 10 mg/mL and a nanofibrous cellulose gel containing 3% cellulose (Kyokuto Pharmaceutical).

Adjustment of the setting of an inkjet printing device for appropriate drop formation

Timing: 1–3 days

The piezo-driven inkjet printing system (LaboJet 500Bio, Microjet Corporation, Figure 1A) controls the formation of droplets by mechanical impact applied to the nozzle tip. This step sets a condition that approximately 10 pL of the enzyme solution is ejected per shot. Finding an appropriate voltage

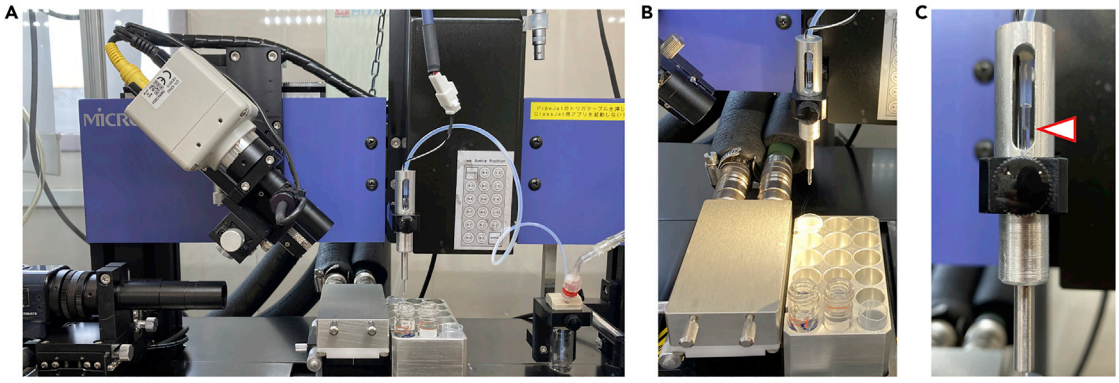

Figure 1. Setup of inkjet patterning device for SPOT

(A) Photograph of the entire device.

(B) Sample holder.

(C) Observation window of the inkjet head. The arrow indicates the middle of the glass capillary where samples should be aspirated.

and pulse width of the piezoelectric actuator that leads to a proper droplet formation is essential. General information on inkjet patterning can be found in several references.[5–7]

Note: The characteristics of the droplet formation (e.g., droplet size, shapes, and flying velocity profiles) are significantly affected by the viscosity and surface tension of the liquid, voltage pulse conditions of the piezo actuator, and even printhead lots (surface treatment of a head nozzle). Standard settings are provided in the manufacturer's instructions, but the condition should be adjusted according to experimental systems.

Note: All solutions (water, enzyme solution, and washing detergent) are passed through a 0.45 μm filter before use to avoid clogging the head nozzle.

1. Power on the system before the experiment, as it takes approximately 15 min for the pressure controller to stabilize. Negative pressure is set to -1.0 kPa.
2. Verify that the values in the manufacturer's specifications can be reproduced when tested with water.
   a. Fill a container (mini vial, 0.3 mL) with filtered water and place it in the tube tray of the sample stage (Figure 1B).
   b. Aspirate water through the nozzle and glass capillary halfway up the observation window (Figure 1C).
      i. Set aspiration time and hold time after aspiration to ~30 s and ~10 s, respectively, on the aspirating/dispensing control software panel.
   c. Check the default settings on the control software panel of the piezo actuator. Example settings are shown in Table 1.

Table 1. Example settings of the piezo actuator

Head serial number	12BBF0503		10CIF0234	
Solution	H_2O	Meicelase 10 mg/mL	H_2O	Meicelase 10 mg/mL
Applied pulse voltage (V)	52	50	34	35
1st pulse width (μs)	110	110	154	120
Rest pulse width (μs)	30	20	30	24
2nd pulse width (μs)	25	45	50	33
Droplet velocity (m/s)	7.5	6.1–6.7	5.7	ND
Drop volume (pg/shot)	16.5	8.93 ± 0.74	26.5	13.17

d. Verify ejection stability in the droplet observation panel.
   i. Ensure that droplets are flying straight in continuous dispensing mode, as seen in Methods video S1.
   ii. If issues are encountered with the proper ejection of drops from the inkjet nozzle, refer to troubleshooting 1, 2, and 3 for possible solutions.
e. Check droplet velocity in the droplet observation panel: adjust the delay time to obtain the position information of a droplet at two measurement points, and calculate its drop velocity.
   i. The drop velocity is preferably in the range 4–8 m/s. Usually, 2–3 droplets are formed in one shot without satellite droplets (Methods video S1).
f. Drain water out of the capillary.

Note: The conditions listed are examples optimized for our equipment. Please adjust the condition settings accordingly depending on the equipment to be used.

3. Adjust the operating condition such that an enzyme solution (Meicelase, 10 mg/mL) is dispensed at approximately 10 pL/shot.
   a. Prepare a 10 mg/mL Meicelase solution in water.
      i. Filter approximately 200 μL of the enzyme solution using a 0.45 μm filter, and place the filtered solution in a container (Mini vial, 0.3 mL).
   b. Set the container on the tube tray of the sample stage and aspirate the enzyme solution to a glass capillary halfway up the observation window.
      i. Set the aspiration and hold times to approximately 50 and 10 s, respectively.
   c. Verify ejection stability in the droplet observation panel.
      i. Make sure that droplets are flying straight in continuous dispensing mode, as seen in Methods video S1.
      ii. If issues are encountered with the proper ejection of drops from the inkjet nozzle, refer to troubleshooting 1, 2, and 3 for possible solutions.
   d. Change settings of the piezo actuator. The example conditions are shown in Table 1.
   e. Collect 100,000 shots of the Meicelase solution (10 mg/mL) in a pre-weighed container and measure the weight using an analytical balance.
   f. Repeat step e and take the average of five measurements.

⚠ CRITICAL: Find conditions that do not generate satellite droplets. See Methods videos S1 and S2 for good and bad examples, respectively. troubleshooting 4.

Pause point: After adjusting the inkjet conditions, experiments (major steps) can be conducted on another day. The inkjet head should be washed to avoid clogging.

Note: To clean the inkjet head, soak it in a neutral detergent solution for 10 min, rinse it with water 3–4 times, and finally clean it with ethanol.

Synchronizing the reference point of the laser microscope and inkjet printing device

Timing: 1 day

Align the position of the nanofibrous cellulose gel on the inkjet patterning device and laser microscope stage. This step enables the drop points printed by the inkjet patterning device to be easily found at the specified coordinates by the laser microscope.

4. Attach a double-sided tape on the microscope stage and fix a glass slide on the tape (Figure 2A). Mark the setting position of the glass slide.

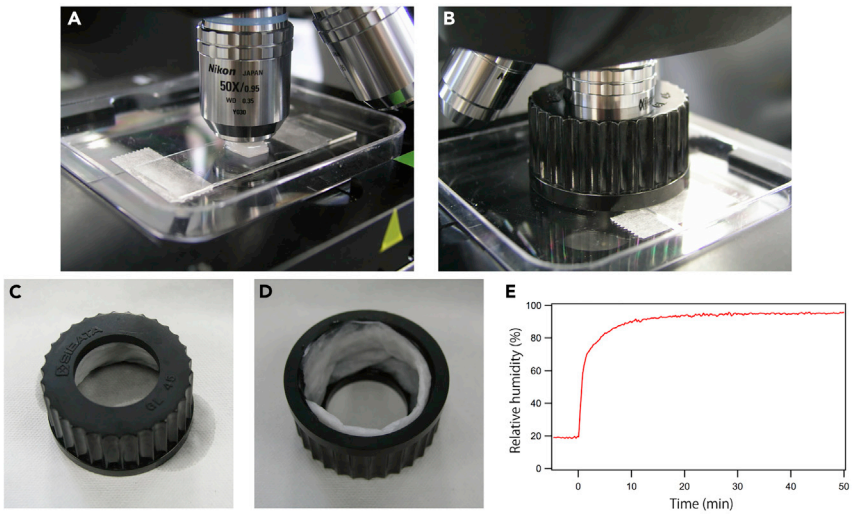

Figure 2. Setup of laser microscope for SPOT

(A) Set-up of the nanofibrous cellulose on the stage. (B) Installation of the humidifying chamber. Covering the sample and objective lens. A lens heater will be mounted on top of the humidifying chamber. (C) Screw top cap. (D) Screw top cap with a wet wipe. (E) Relative humidity inside the humidifying chamber.

5. Use a marker pen to mark the point on the glass slide to place the nanofibrous cellulose gel directly below the objective lens of the laser microscope.
6. Remove the glass slide from the microscope and place nanofibrous cellulose gel on the marked position of the glass slide.
7. Set the glass slide on the corner of the stage of the inkjet patterning device.
8. The printing area should be the center area of the nanofibrous cellulose gel. Record the starting point of printing as the reference point of the inkjet patterning device.
9. Print cellulase solution onto the nanofibrous cellulose gel and wait for several minutes until a pit grows to a size that can be seen with a laser microscope.
10. Find the pit with the laser microscope.
11. Record the coordinates of the pit by the coordinate recording function of the motorized stage of the laser microscope.

Note: Printing drops at the target coordinates with the inkjet is not always possible because the drop may not fly straight due to static electricity.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Exedia® Cellulose plate -original-	Kyokuto Pharmaceutical	N/A
Microcrystalline cellulose (Avicel)	Merck	102331;CAS 9004-34-6
Calcium thiocyanate tetrahydrate	Kanto Chemical	07112-01
Methanol	FUJIFILM Wako Pure Chemical	138-01831
Meicelase (mixture of cellulases from *Trichoderma viride*)	Meiji Seika	N/A
Software and algorithms		
Scanning probe image processor (SPIP)	Image Metrology	https://www.imagemet.com
RhManager, a managing software for Hygrochron	KN Laboratories	https://www.kn-labs.com/rhmanager_download_e.htm

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Laser scanning confocal microscope, VK-9700 Generation II	Keyence	N/A
Inkjet patterning device, LaboJet-500Bio	Microjet Corporation	http://www.inkjet-bio.com/eng/01_en_products/LJ-Bio/index.html
Inkjet printhead, GlassJet head for bio-use	Microjet Corporation	IJHBS-10
Lens heater	Tokai Hit	N/A
Screw-Top Cap with Hole 29.5 mm	Sibata Scientific Technology	016060-045A
Mini vial 0.3 mL	Osaka Chemical	MV-03
Analytical balance	Mettler Toledo	AG135
Belly dancer orbital shaker	Stovall	N/A
Clean Ace (s), non-phosphorus, cleaning concentration liquid	As One	4-078-01
BEMCOT M-1, fine-grade wipes	Asahi Kasei	N/A
Millex-HV syringe filter unit, 0.45 μm	Millipore	SLHV013SL
Hygrochron temperature and humidity logger	KN Laboratories	N/A

MATERIALS AND EQUIPMENT

Nanofibrous cellulose

The details of the preparation protocol and characterization of nanofibrous cellulose can be found in a previous study.[8] To prepare the nanofibrous gel consisting of the porous network of crystalline nanofibers (cellulose-II, 44.5% crystallinity), Avicel cellulose (microcrystalline cellulose for thin-layer chromatography; Merck) is dissolved at a concentration of 3 wt% in a hot, saturated solution of calcium thiocyanate (approximately 60 wt%) in water. The solution is allowed to cool to room temperature, during which the cellulose is regenerated and forms the gel spontaneously. To remove the calcium thiocyanate, the gel is washed with methanol and water. Ready-made nanofibrous cellulose gel plates can be obtained from Kyokuto Pharmaceutical.

Cellulase

Nanofibrous cellulose contains a substantial amount of crystalline domain (cellulose-II, 44.5% crystallinity). Endoglucanase alone cannot hydrolyze nanofibrous cellulose.[1] A cellulase capable of degrading crystalline cellulose can be used for quantitative analysis. This protocol uses Meicelase, a mixture of fungal cellulases from *Trichoderma viride*.[9]

Humidifying chamber

A humidifying chamber is used during optical profilometric measurements of the pit to minimize the evaporation of water from the wet nanofibrous cellulose matrices (Figure 2B). Because the objective lens moves vertically (z-direction) during measurement, a screw-top cap of diameter 29.5 mm (Figure 2C) is used as the chamber. A wet wipe was packed inside the cap immediately before the measurements (Figure 2D). The relative humidity inside the chamber reaches ~90%; data in Figure 2E were monitored by placing the Hygrochron humidity logger inside the humidifying chamber at room temperature in the presence of the lens heater. Without the humidifying chamber, the height of the cellulose surface drops to 140 μm in 30 min at room temperature.

STEP-BY-STEP METHOD DETAILS

Preparation of cellulose gel: Solvent exchange to the reaction buffer

Timing: 3–5 h

Nanofibrous cellulose gel plates (with diameter and thickness of 9 cm and 5 mm, respectively) contain water after preparation. The water should be replaced with the appropriate buffer for the enzymatic reaction.

Note: All subsequent experiments are performed at room temperature (22°C–25°C) with normal humidity (14%–20% relative humidity).

1. Solvent exchange of the nanofibrous cellulose.
   a. Prepare 2× buffer of the target reaction buffer. In this protocol, 0.2 M acetate buffer (pH 4.8) is prepared.
   b. Pour 20 mL, that is, the same volume as the nanofibrous cellulose plate, of 2× buffer into a Petri dish containing the nanofibrous cellulose plate.
   c. Leave the plate for 1–4 h with gentle shaking on an orbital shaker.
   d. Remove excess buffer with a pipette. Nanofibrous cellulose is stored in airtight Tupperware containers at 4°C.
2. Cut the disc-shaped cellulose plate into square pieces (1–3 cm across) and place a piece on a glass slide.
3. Air dry the cellulose plate for 20–30 min to remove excess water on the surface.
   a. During air drying, enter the thickness value of the nanofibrous cellulose and glass slide into the material thickness parameter in the stage control panel of the inkjet device.

Note: This setting is used as reference in the subsequent inkjet printing experiments to control the distance between the nozzle tip and gel surface.

⚠ CRITICAL: Maintaining standardized air-drying conditions is critical to obtain consistent pit-formation results. Air drying considerably affects the diffusion of enzymes into cellulose matrices; thus, pit sizes vary depending on the air-drying condition; for example, longer drying results in larger pits. See also troubleshooting 5.

Note: An air-drying time of 20–30 min is recommended. As the enzyme deposition by the inkjet printing device is sometimes unstable, as in troubleshooting 1, 2, and 3, this leaves enough margin for troubleshooting before the next inkjet printing experiment. Proceed to the next steps while air-drying, and keep the time of enzyme deposition (major step 6) constant after removing the gel from the airtight container (major step 2).

Note: When the solvent exchange was tested with Luria–Bertani broth, the exchange was almost completed within 2 h.[8]

Deposition of cellulase on nanofibrous cellulose by inkjet printing

Timing: ~10 min

A solution containing 10 mg/mL cellulases (Meicelase) is deposited onto a flat surface of a nanofibrous cellulose gel using an inkjet patterning device (LaboJet 500Bio) equipped with an IJHBS 10 inkjet head. The amount of deposited solution is controlled by repeatedly depositing the solution in the same place.

Note: Turn on the laser microscope for 60 min at minimum before use.

Note: The condition used in this protocol was 8.93 ± 0.74 pL of the 10 mg/mL cellulase solution per shot (Table 1).

4. Mount the slide glass with nanofibrous cellulose on the sample stage base.
5. Settings of the patterning methods (Examples are shown in Table 2).
   a. Set the distance from the nozzle tip of the inkjet print head to the surface of the nanofibrous cellulose gel to 1 mm (Gaps in Table 2).
   b. Set the interval between the droplet (dot spacing) to 400 μm.

Table 2. An example setting of patterning methods of inkjet printing

Step no.	Methods	Coordinates dX (μm)	dY(μm)	Gaps (μm)	Numbers of shots	Numbers of repeats in Y	Offsets Y (μm)
1	Droplet observation						
2	Patterning	-400	0	1000	1	9	400
3	Patterning	0	0	1000	10	9	400
4	Patterning	400	0	1000	8	9	400
5	Patterning	800	0	1000	6	9	400
6	Patterning	1200	0	1000	4	9	400
7	Patterning	1600	0	1000	2	9	400
8	Patterning	2000	0	1000	1	9	400
9	Patterning	2500	0	1000	10	9	500
10	Patterning	3000	0	1000	8	9	500
11	Patterning	3500	0	1000	6	9	500
12	Patterning	4000	0	1000	4	9	500
13	Patterning	4500	0	1000	2	9	500
14	Patterning	5000	0	1000	1	9	500

c. Set the number of shots to be deposited; for example, 2 (0.18 ng of cellulases), 4 (0.36 ng), 6 (0.72 ng), or 8 shots (0.90 ng).
d. Include the droplet observation method to check the droplet performance (Table 2, step no. 1).
e. Set a test condition outside the print area because the first shot is not stable (Table 2, step no. 2).

6. Start patterning and check the droplet performance.
   a. If issues are encountered with the proper ejection of drops from the inkjet nozzle, refer to troubleshooting 1, 2, and 3 for possible solutions.
   b. The reaction start time is defined as the time immediately after the device starts to move for patterning.

Note: To assess reproducibility, major steps 2–6 and the laser profilometry experiments (major steps 7–15) are repeated 10 times for each condition.

Note: Patterning of multiple spots under the same drop conditions is recommended owing to the possible existence of surface profile disorder, scratches, or dust adhering to the surface. Even if only a single spot is measured using a laser microscope, a spot with a smooth surface can be selected.

Optional: The above protocol is for tracking the growth of a spot over time. If the purpose of the measurement is to observe multiple spots without the need to take a time-course of enzymatic activity, variations can be made in the amount of enzyme drops in each patterning experiment.

Note: Spacing intervals of the points are set to 400 μm to ensure that the points are spaced with sufficient distance to generate a flat baseline. See also expected outcomes and Figure 3.

Quantification of pits using a laser scanning confocal microscope

Timing: ~30 min

Pits on the surface of nanofibrous cellulose matrices are quantified using a laser scanning confocal microscope operated in reflection mode. Three-dimensional height maps of the pitted surface are obtained in a non-destructive manner.

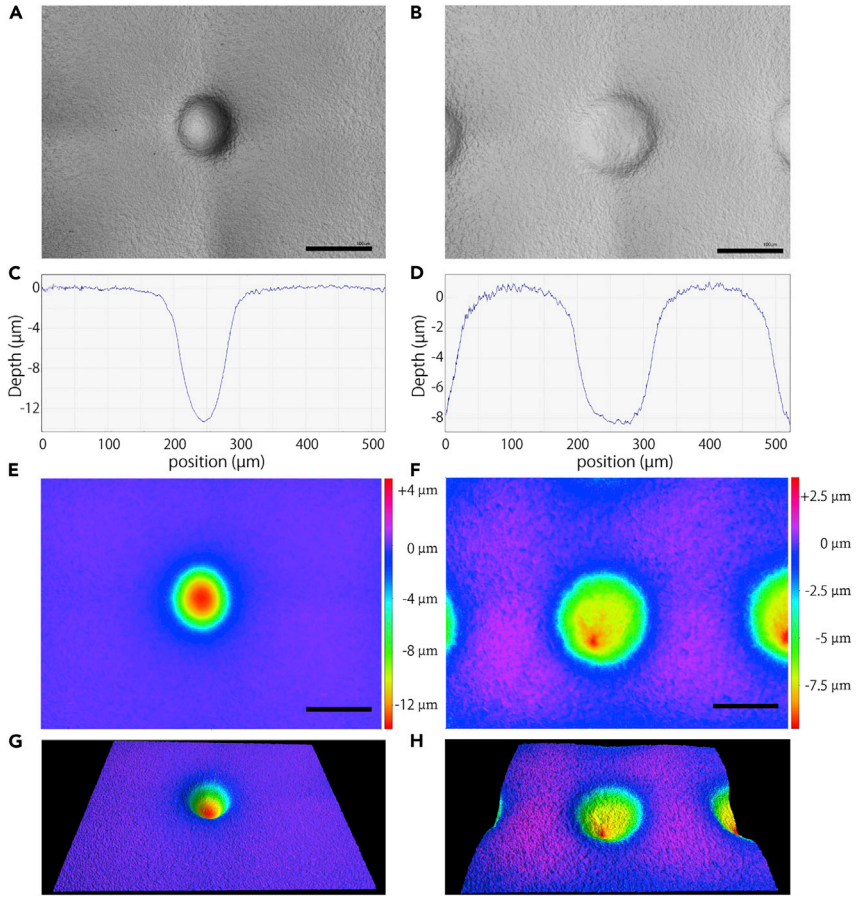

Figure 3. Effects of pit intervals on the baseline

(A–H) Pits with intervals of 400 μm (A, C, E, and G) and pits with intervals of 300 μm (B, D, F, and H). A successful image shows a flat baseline in its cross-sectional profile (C); however, baselines are ambiguous when the intervals are too narrow (D). Additionally, the pit with intervals of 300 μm is large in diameter but shallow; in addition, the pit does not exhibit a gaussian-like cross-sectional profile. Longer air-drying should be applied to ensure pit formation in the depth direction (troubleshooting 5). Both pits were observed at 29 min after depositing 0.89 (A, C, E, and G) and 1.32 ng (B, D, F, and H) of cellulases. Scale bars: 100 μm.

Note: The microscope uses a laser (λ = 408 nm) to scan the target in the field of view along the X and Y directions (1,024 × 768 pixels) for every single plane. The intensity of reflected light from the surface, *I*, is measured by a photomultiplier. Measurements are repeated to obtain a stack of images as the objective lens moves in the depth (*z*) direction at a specified pitch. A focal position of a pixel is given at *z*, where the strongest *I* is recorded. A 3D height map is generated by locating the focal positions of all pixels in an image.

Note: A 50× objective lens (N.A. 0.95, a field of view: 270 μm × 202 μm) is used in this protocol. A 20× objective lens (N.A. 0.46, a field of view: 675 μm × 506 μm) is also usable; however, the resolution is insufficient for quantifying enzymatic pit formation.

Note: Imaging is performed using the panorama mode with an aid of automatic control by the motorized electric stage. Four images (2 × 2 grids) are collected for each spot and computationally joined together to yield an image covering an area larger than a single field of view.

Note: The space inside the pit remains dry because the excess water released during the disruption of the 3D network is re-absorbed by the surrounding matrix.

7. A lens heater is attached to the objective lens to prevent water condensation on the lens surface.
   a. Set the heater temperature to 40°C; > 10 min is required to reach the set temperature.
8. Move to the spot position to be measured.
9. Mount nanofibrous cellulose on the stage of the microscope (Figure 2A); place the glass slide to the defined place on the stage using double-sided tape.
10. Place the humidifying chamber on the stage to cover the nanofibrous cellulose and objective lens (Figure 2B).
11. Adjust the focal position to get a view of the target to be examined.
12. Determine the *z* range to be scanned: move the peak (focal spot) to the top and bottom of a target and save the peak information in the memory.
13. Take panoramic images of 4 views (2 × 2 grids).
14. Continue measurements by repeating the major steps 12 and 13 until the end of the incubation (30 min).
15. Export image files. The panorama images are computationally joined together.

Note: The surface profile around the pit is measured extensively because a flat surface area is necessary to obtain a reference surface for accurate volume calculations. See also expected outcomes and Figure 3.

Note: Scan time is ~1 min for each image. Shallow pits (reaction time 5 min) require ~3 min per four images, whereas deeper pits (reaction time 30 min) require ~5 min per four images.

Quantification and statistical analysis of pit volume

Timing: ~5 min

Pit volumes are calculated from the 3D height map using a scanning probe image processor (SPIP ver. 6.13). SPIP contains many generic analytical and visualization tools. The specialized tools for plane correction are particularly useful for quantitatively analyzing the pit volume.

Note: The roughness of the nanofibrous cellulose surface was determined to be 0.2–0.4 μm using the root mean-square deviation (R_q) of the average amplitude in the height direction. To eliminate errors due to surface irregularities, set the depth threshold to –1 μm. Data of the surface areas above the threshold are excluded from the calculation. The appropriate threshold for the detection of shallow pits can be adjusted according to the specific experimental conditions. If very shallow pits need to be detected with high sensitivity, setting the threshold to 0.5 μm may be appropriate.

16. Import an image file into the SPIP software.
17. Polynomial plane correction (flattening).
    a. Select the pit by Area of Interest marking tools (Circle markers). The fit surface for plane correction is calculated based on the data outside the Area of Interest in the next step b.
    b. Open the Plane Correction Dialog, apply the setting in Table 3, and perform plane correction.
18. The SPIP software automatically generates the height distribution histogram of the image.
    a. Select volumes below the threshold (-1 μm) in the Histogram window.
    b. The SPIP software integrates the volume of the selected area.
19. Multiply the integrated volume data below the threshold (-1 μm) by pixel dimension (0.3 μm × 0.3 μm for a 50× objective) and fiber density (0.03 g cm^{-3} for 3% nanofibrous cellulose).

Note: Since the surface of the nanofibrous cellulose gel is slightly undulated rather than flat, it is recommended to use image analysis software with plane correction tools of higher-order flattening.

Table 3. Settings of the plane correction algorithms

Methods	Selected options	Descriptions
Global correction	Polynomial fit	Remove the tilt and unwanted curvature of the image by subtracting a fitted image calculated by the least mean-square fit.
Estimation volume	Frame region	The plane fit will be based only within the specified outer frame.
Line-wise correction	OFF	
Z offset method	Bearing height to zero	The image is leveled by setting the most dominant height value (highest frequency in height distribution histogram) to zero.

Note: Our histogram data is available in the Figure S8 in a previous study.[1]

EXPECTED OUTCOMES

Successful images are generated with flat baselines (Figure 3A). If the intervals of each spot are too narrow, the baseline is lower than the true value, and the volume calculation becomes inaccurate (Figure 3B).

The measured mass of hydrolyzed cellulose increased linearly with the reaction time up to 15 min, and the growth slowed thereafter.[1] The initial hydrolysis rates, which are obtained from the slope of linear fits to the data points within 15 min, are between 60 and 180 pg min^{-1} and proportional to the amount of deposited cellulases.[1]

LIMITATIONS

The morphology and hydrolyzed volumes of the pits are strongly affected by air-drying of the cellulose gel because the treatment alters the diffusion behavior of enzymes. Additionally, heterogeneity in nanofibrous cellulose leads to larger errors. Repeated measurements are required to obtain quantitative, reproducible results. To obtain an average value for a single reaction condition, it is common practice to take 10 measurements and calculate the mean.

The crystalline structure of nanofibrous cellulose, such as the degree of crystallinity and crystalline polymorph, cannot be controlled in the present method of preparing nanofibrous cellulose.

It is worth noting that, depending on the printing parameters, mechanical stress (compressive stress in the print head chamber and shear stress at the nozzle) may affect the activity and stability of enzymes.[10] This effect has not yet been studied for cellulase using this method, but it is a potential consideration.

The preparation of the porous and homogeneous substrate with a flat surface is essential for the application of SPOT for other enzymatic reactions. Preparation methods of porous nanofibrous matrices are not always available, but several examples include polysaccharides such as chitin,[11] proteins such as gelatin,[12] and biodegradable synthetic polymers such as poly(L-lactic acid).[13]

TROUBLESHOOTING

Problem 1

Air bubbles in the nozzle (before you begin steps 2 and 3 and Major step 6).

Potential solution

Air may be introduced into the inkjet printhead while filling the enzyme solution, setting the negative pressure, and wiping the nozzle tip with a wet wipe. Replace the enzyme solution in the printhead. Set a slower aspiration speed and longer hold time after aspiration when filling the nozzle with the enzyme solution.

Problem 2
The nozzle tip dries out and clogs (before you begin steps 2 and 3 and Major step 6).

Potential solution
When using highly concentrated enzyme solutions, the tip of the inkjet nozzle tends to dry out and clog. Wipe the nozzle tip frequently with a wet wipe and use the refreshing mode of the inkjet device to automatically eject drops while waiting.

Problem 3
If the unstable ejection is still encountered and the solutions for problems 1 and 2 do not work, there are additional steps that can be taken (before you begin steps 2 and 3 and Major step 6).

Potential solution
Static electricity may affect the ejection stability; therefore, static elimination by an anti-static instrument should be conducted. In addition, check the settings of meniscus pressure and clean the inkjet nozzle.

Problem 4
Satellite formations around the pit (before you begin step 3).

Potential solution
Bring the nozzle and nanofibrous cellulose gel close together when depositing the enzyme solution. Eliminate static electricity using an anti-static instrument.

Problem 5
Spots become larger but do not grow in depth (expected outcomes, Figures 3B, 3D, 3F, and 3H).

Potential solution
Insufficient air-drying of nanofibrous cellulose gel (Major step 3): set a longer air-drying time.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shigeru Deguchi (shigeru.deguchi@jamstec.go.jp).

Materials availability
All materials generated in this study are available from the lead contact without restriction.

Data and code availability
- This study did not generate datasets.
- This paper does not report original codes.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102066.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.D.; Methodology, M. Tsudome; Investigation, M. Tsudome; Visualization, M. Tsudome, M. Tachioka, S.D.; Formal analysis, M. Tsudume, M. Tachioka, S.D.; Supervision, S.D.; Writing—original draft, M. Tachioka, M. Tsudome; Writing—review & editing, S.D.

DECLARATION OF INTERESTS

JAMSTEC has patents relating to the production and uses of the nanofibrous cellulose, of which S.D. and M. Tsudome are named inventors. S.D. and M. Tsudome have received research support and may profit from the sale of the nanofibrous cellulose, which has been developed by Kyokuto Pharmaceutical Industrial Co., Ltd., Japan, through a joint research agreement with JAMSTEC.

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