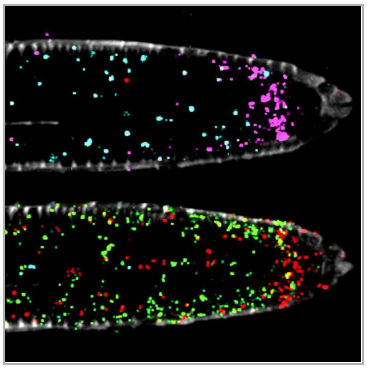

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PHYTOMap in Arabidopsis root tips

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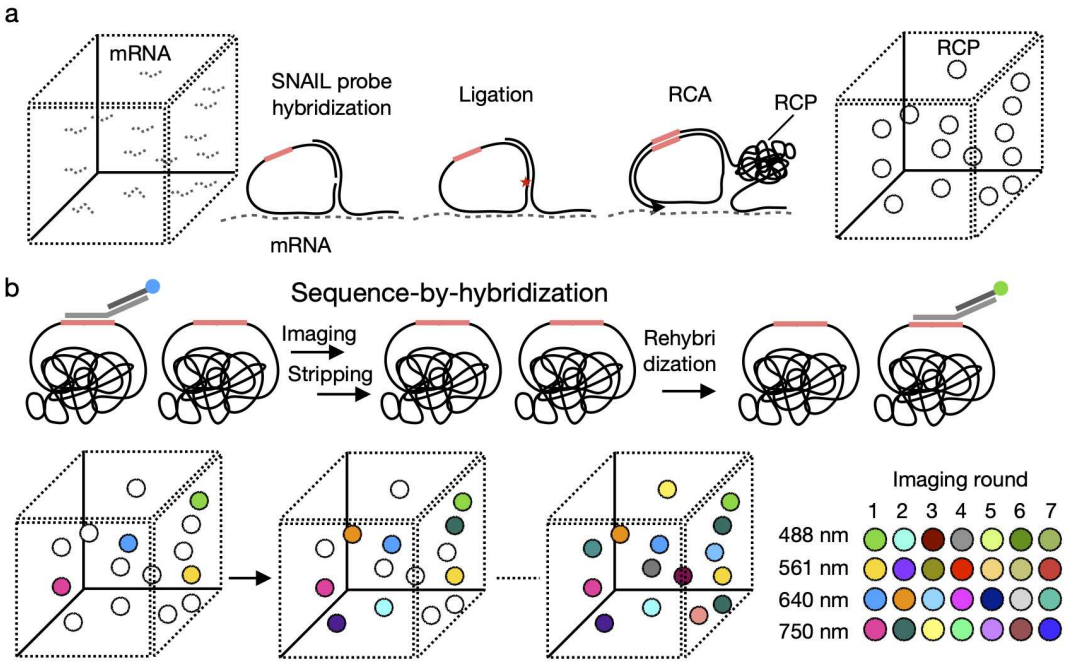
ABSTRACT

Retrieving the complex responses of individual cells in the native three-dimensional tissue context is crucial for a complete understanding of tissue functions. Here, we present PHYTOMap (Plant HYbridization-based Targeted Observation of gene expression Map), a multiplexed fluorescence *in situ* hybridization method that enables single-cell and spatial analysis of gene expression in whole-mount plant tissue in a transgene-free manner and at low cost. We applied PHYTOMap to simultaneously analyze 28 cell type marker genes in *Arabidopsis* roots and successfully identified major cell types, demonstrating that our method can substantially accelerate the spatial mapping of marker genes defined in single-cell RNA-seq datasets in complex plant tissue.



PHYTOMap principles. a, Target mRNA molecules are hybridized by DNA probes (SNAIL probes) that harbor mRNA species-specific barcode sequences (pink bars). The barcode-containing DNA probes are circularized by ligation and amplified *in situ* by rolling circle amplification (RCA). b, Amplified DNA barcodes are detected by sequence-by-hybridization. Different fluorescent probes target four

OPEN ACCESS

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Protocol status: Working
We use this protocol and it's working

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PROTOCOL integer ID: 81174

Keywords: In situ hybridization, plant biology, spatial transcriptomics

DNA barcodes for each imaging round. After the imaging, fluorescent probes are stripped away, and another set of four genes is targeted.

[Reagents]

DPBST
0.1% (vol/vol) Tween-20 in 1x DPBS

DPBSTR
DPBST + 1:100 SUPERaseIN

Cell wall digestion enzyme solution (CWDES; 10x stock)
250 mg macerozyme, 250 mg cellulase, 500 mg pectinase in 50 mL Nuclease-free water. Filter sterilize (0.22 µm filter) and store aliquots of 1 mL at -20°C.

Proteinase K buffer

	A	B
	Reagent	Amount
	1M Tris-HCl (pH 8.0)	5 mL
	0.5 M EDTA (pH 8.0)	5 mL
	Nuclease Free Water	up to 50 mL

FAA

	A	B
	Reagent	Amount
	32% formaldehyde	450 µL
	Acetic acid	50 µL
	Ethanol	500 µL

GUIDELINES

See the following paper for more information on PHYTOMap.

PHYTOMap: Multiplexed single-cell 3D spatial gene expression analysis in plant tissue
Tatsuya Nobori, Marina Oliva, Ryan Lister, and Joseph R. Ecker, bioRxiv, 2022
DOI: 10.1101/2022.07.28.501915

- To avoid RNase contamination, special precautions are necessary when handling samples in this protocol. It is advisable to allocate a dedicated area and equipment specifically for RNA work, and to clean them with commercial RNase and DNAse inactivating agents before wiping them with ethanol.
- Probes have to be accurately designed. Detailed information on probe design is available in the publication above.

MATERIALS

[Materials]

- Poly-D-Lysine coated dish (MatTek, P35GC-1.5-14-C)
- T4 DNA Ligase (Thermo Scientific, EL0011)
- EquiPhi29 DNA Polymerase (Thermo Scientific, A39391)
- SUPERaseIn RNase Inhibitor (Invitrogen, AM2696)
- Aminoallyl dUTP (AnaSpec, AS-83203)
- Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma, D8662)
- BSA, molecular biology grade (New England Biolabs, B9000S)
- dNTPs (New England Biolabs, N0447S)
- Fluorescent Brightener 28 disodium salt solution (Sigma, 910090)
- Formaldehyde Solution for Molecular Biology, 36.5-38% in Water (Sigma, F8775)
- Triton-X (Sigma, 93443)
- Proteinase K (Invitrogen, 25530049)
- Nuclease-Free Water (Invitrogen, AM9937)
- BS(PEG)9 (Thermo Scientific, 21582)
- 20 ×SSC buffer (Sigma-Aldrich, S6639)
- Ribonucleoside vanadyl complex (RVC) (New England Biolabs, S1402S)
- Formamide (Sigma, F9037)
- Tris, pH 8.0, RNase-free (Invitrogen, AM9855G)
- EDTA, pH 8.0, RNase-free (Invitrogen, AM9260G)
- Gel Slick™ Solution (Lonza, 50640)
- Cellulase (Yaklut, YAKL0013)
- Macerozyme (Yakult, YAKL0021)
- Pectinase (Fisher Scientific, ICN19897901)

[Reagents]

DPBST

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DPBSTR
DPBST + 1:100 SUPERaseIN

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Proteinase K buffer

	A	B
	Reagent	Amount
	1M Tris-HCl (pH 8.0)	5 mL
	0.5 M EDTA (pH 8.0)	5 mL
	Nuclease Free Water	up to 50 mL

FAA

	A	B
	Reagent	Amount
	32% formaldehyde	450 µL
	Acetic acid	50 µL
	Ethanol	500 µL

SAFETY WARNINGS

See safety data sheets for proper chemical handling, precautionary measures and waste disposal.
Obey all local regulations/guidelines for handling and disposal of used reagents and and solutions containing reagents mixed in.

Formamide:
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
Suspected of causing cancer.
May damage fertility or the unborn child.
May cause damage to organs (Blood) through prolonged or repeatedexposure if swallowed.

Formaldehyde:
Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.
May cause cancer.
Toxic if swallowed, in contact with skin or if inhaled.
Causes severe skin burns and eye damage.
May cause an allergic skin reaction.
May cause respiratory irritation.
Suspected of causing genetic defects.
Causes damage to organs (Eyes).

Sampling

1 Cut Arabidopsis root tips (approximately 1 cm) with razor blade and place them on a Poly-D-Lysine coated dish. The tissue should adhere to the dish well. 2m

Tissue Fixation

2 Incubate the tissue in 100 µL of FAA (see MATERIALS) at Room temperature for 01:00:00 . 1h

Note

If most of the root tips detach from the dish, it is necessary to optimize the mounting process for the samples.

3 Wash the tissue successively for 00:10:00 at Room temperature with 100 µL 70% EtOH, 90% EtOH, and twice with 100% EtOH. 10m

4 Wash the tissue twice with 100 µL 100% MeOH for 00:10:00 and leave the tissue in MeOH after the second wash at -20 °C Overnight. 40m

Tissue Permeabilization

5 Rehydrate the tissue by 00:05:00 successive washes with 100 µL 75%, 50%, 25% MeOH in DPBST. 5m

6 Incubate the tissues in 100 µL 1x CWDES (see MATERIALS) On ice for 00:05:00. 5m

	A	B
	Reagent	Amount
	10x CWDES	10 µL
	SUPERase IN	1 µL
	DPBST	89 µL

1x CWDES

7 Remove the CWDES and add 100 µL fresh & cold 1x CWDES. Then, incubate the tissue at Room temperature for 00:30:00. 30m

8 Wash the tissue twice with 100 µL DPBSTR (see MATERIALS).

9 Fix the tissue by incubating in 100 µL 10% (v/v) formaldehyde in DPBST at Room temperature for 00:30:00. 30m

10 Wash the tissue twice with 100 µL DPBSTR at Room temperature (00:01:00 each). 1m

11 Incubate the tissue in 100 µL digestion solution at 37 °C for 00:30:00. 30m

	A	B
	Reagent	Amount
	Proteinase K buffer (see MATERIALS)	99 µL
	Proteinase K	1 µL

Digestion solution

12 Wash the tissue twice with 100 µL DPBSTR at Room temperature (00:01:00 each). 1m

13 Fix the tissue by incubating in 100 µL 10% (v/v) formaldehyde in DPBST at Room temperature for 00:30:00. 30m

14 Wash the tissue twice with 100 µL DPBSTR at Room temperature (00:05:00 each). 5m

Gene Specific Probe Hybridization

15 Mix gene specific probes at the concentration of 5 nM per oligo.

16 Heat the probe mixture at 90 °C for 00:03:00 and let it cool down at Room temperature . 3m

17 Incubate the tissue in the hybridization mixture at 40 °C for 03:00:00 or Overnight 6h

	A	B
	Reagent	Amount
	20xSSC	10 µL
	Formamide	30 µL
	10% Triton-X	10 µL
	200 mM RVC	10 µL
	SUPERase IN	1 µL
	Probe mix (500 nM per oligo)	2 µL
	Nuclease Free Water	37 µL

Hybridization mixture

Note

The design of probes is described in our paper.

18 Wash the tissue twice with 100 µL DPBSTR at 37 °C for 00:30:00 . 30m

19 Wash the tissue twice with 100 µL 4xSSC in DPBSTR at 37 °C for 00:30:00 . 30m

	A	B

	A	B
	Reagent	Amount
	20xSSC	20 µL
	DPBST	79 µL
	SUPERase IN	1 µL

4xSSC in DPBSTR

20 Rinse the tissue with 100 µL DPBSTR at Room temperature.

Ligation

21 Incubate the tissue in 100 µL ligation mixture WITHOUT ligase On ice for 00:05:00. 5m

	A	B
	Reagent	Amount
	10x ligation buffer	10 µL
	BSA (2mg/ml)	0.5 µL
	SUPERase IN	1 µL
	Nuclease Free Water	88.5 µL

Ligation mixture without ligase

22 Incubate the tissue in 100 µL ligation mixture WITH ligase at Room temperature Overnight.

	A	B
	Reagent	Amount
	10x ligation buffer	10 µL
	BSA (20 mg/ml)	0.5 µL

	A	B
	SUPERase IN	1 µL
	T4 DNA ligase	2 µL
	Nuclease Free Water	86.5 µL

Ligation mixture

Rolling Circle Amplification (RCA)

23 Wash the tissue with 100 µL DPBSTR for 00:05:00. 5m

24 Incubate the tissue in 100 µL RCA mixture WITHOUT equiPhi29 DNA polymerase On ice for 00:05:00. 5m

	A	B
	Reagent	Amount
	10x equiPhi29 DNA polymerase buffer	10 µL
	10 mM dNTP	2.5 µL
	4 mM aminoallyl-dUTP	0.5 µL
	BSA (20 mg/ml)	0.5 µL
	DTT (100 mM)	1 µL
	SUPERase IN	1 µL
	Nuclease Free Water	84.5 µL

RCA mixture without equiPhi29 DNA polymerase

25 Incubate the tissue in 100 µL RCA mixture WITH equiPhi29 DNA polymerase at 37 °C Overnight. 5m

	A	B

	A	B
	Reagent	Amount
	10x equiPhi29 DNA polymerase buffer	10 µL
	10 mM dNTP	2.5 µL
	4 mM aminoallyl-dUTP	0.5 µL
	BSA (20 mg/ml)	0.5 µL
	DTT (100 mM)	1 µL
	SUPERase IN	1 µL
	equiPhi29 DNA polymerase	5 µL
	Nuclease Free Water	79.5 µL

RCA mixture with equiPhi29 DNA polymerase

26 Wash the tissue twice with 100 µL DPBSTR for 00:10:00. 10m

Post-Amplification Fixation

27 Incubate the tissue in 100 µL BS(PEG9) solution at Room temperature for 01:00:00. 1h

	A	B
	Reagent	Amount
	DPBST	98 µL
	BS(PEG9) stock	2 µL

BS(PEG9) solution

Note

BS(PEG9) stock is made by adding 465 µL DMSO into a vial of 100 mg BS(PEG9) and is stored desiccated at -20°C.

28 Aspire BS(PEG9) solution and incubate the tissue in 100 µL 1 M Tris-HCl pH 8.0 at Room temperature for 00:30:00. 30m

29 Rinse the tissue in 100 µL DPBST.

> **Note**
>
> If multiple rounds of imaging are not necessary, gel embedding may be skipped. However, it is important to note that without gel embedding, there is a higher risk of tissue detachment or movement between imaging rounds. Therefore, it is recommended to carefully handle the tissue.

Gel Embedding

30 Incubate the tissue in 100 µL monomer solution On ice for 00:30:00. 30m

	A	B
	Reagent	Amount
	20% acrylamide	20 µL
	2% bis-acrylamide	10 µL
	2xSSC	70 µL

Monomer solution

31 Aspire the monomer solution and add 50 µL gelling solution. Place a Gel Slick-coated glass coverslip on top of the tissue, and carefully aspirate any excess gelling solution. Incubate the tissue at Room temperature for 01:00:00 -- 02:00:00 until the gel solidifies. 3h

	A	B
	Reagent	Amount
	Monomer solution	48.9 µL
	10% APS stock	1 µL

	A	B
	TEMED	0.1 µL

Gelling solution

> **Note**
>
> Coating a coverslip with Gel Slick:
> Add Gel Slick Solution onto the coverslip, then wipe gently with a Kimwipe to spread the Gel Slick Solution.

32 Carefully remove the coverslip with forceps, and wash the tissue with 100 µL DPBST for 00:05:00. 5m

33 Incubate the tissue in ClearSee until imaging.

Target Detection with Sequence-By-Hybridization

34 Wash the tissue twice with 100 µL 2xSSC for 00:01:00. 1m

35 Incubate the tissue in 100 µL Bridge probe mixture at Room temperature for 01:00:00. 1h

	A	B
	Reagent	Amount
	2x hybridization buffer	50 µL
	Bridge probes (10uM stock)	1 µL each (typically 4 probes)
	Nuclease free water	up to 100 µL

Bridge probe mixture

36 Wash the tissue twice with 100 µL 2xSSC for 00:01:00. 1m

37 Incubate the tissue in 100 µL Detection probe mixture at Room temperature for 01:00:00. 1h

	A	B
	Reagent	Amount
	2x hybridization buffer	50 µL
	Detection probes (10uM stock)	1 µL each (4 probes)
	Calcofluor White	1 µL
	Nuclease free water	up to 100 µL

Detection probe mixture

38 Wash the tissue twice with 100 µL 2xSSC for 00:01:00. 1m

39 Wash the tissue with 100 µL ClearSee, and keep it in ClearSee for imaging.

40 Imaging with a confocal microscope.

41 After the imaging, strip the bridge/detection probes by incubating the tissue in 100 µL stripping solution (65% formamide in 2xSSC) at 30 °C for 00:30:00. 30m

	A	B
	Reagent	Amount
	Formamide	65 µL
	20xSSC	10 µL
	Nuclease free water	up to 100 µL

Stripping solution

42 Go to **Step 35** for the next round of imaging.