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Protocol Note

Application of a novel and automated branched DNA in situ hybridization method for the rapid and sensitive localization of mRNA molecules in plant tissues[1]

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- *Premise of the study:* A novel branched DNA detection technology, RNAscope in situ hybridization (ISH), originally developed for use on human clinical and animal tissues, was adapted for use in plant tissue in an attempt to overcome some of the limitations associated with traditional ISH assays.
- *Methods and Results: Zea mays* leaf tissue was formaldehyde fixed and paraffin embedded (FFPE) and then probed with the RNAscope ISH assay for two endogenous genes, phosphoenolpyruvate carboxylase (*PEPC*) and phosphoenolpyruvate carboxykinase (*PEPCK*). Results from both manual and automated methods showed tissue- and cell-specific mRNA localization patterns expected from these well-studied genes.
- *Conclusions:* RNAscope ISH is a sensitive method that generates high-quality, easily interpretable results from FFPE plant tissues. Automation of the RNAscope method on the Ventana Discovery Ultra platform allows significant advantages for repeatability, reduction in variability, and flexibility of workflow processes.

Key words: branched DNA; in situ hybridization; phosphoenolpyruvate carboxykinase; phosphoenolpyruvate carboxylase; RNAscope; *Zea mays*.

The success of experiments that rely on gene regulation often depends on an understanding of the expression pattern of the mRNA transcripts of interest. However, localization of specific mRNA molecules within large and diverse sample sets is often hampered by the limitations of traditional in situ hybridization (ISH) methods. Classical ISH methods for plant tissue sections usually require many pretreatment steps and posthybridization washes to combat background issues (see, for example, Brewer et al., 2006). Although there have been some efforts to simplify the conventional ISH process (Borlido et al., 2002) and also some studies aimed at streamlining and partially automating the ISH process (Drea et al., 2005), these methods all rely on detecting labeled antisense RNA probes hybridized with endogenous mRNA molecules either directly (e.g., with fluorescence) or with antibody-based detection strategies.

RNAscope ISH (Advanced Cell Diagnostics [ACD], Hayward, California, USA) is an improved method for localizing mRNA molecules more specifically, more rapidly, and with greater sensitivity than previously possible with conventional ISH methods (Wang et al., 2012). RNAscope ISH is a form of branched DNA (bDNA) ISH that also utilizes pairs of 'Z-probes' that are highly specific to target genes and yet are small enough to readily diffuse into tissue sections. Each Z-probe is composed of a unique region of approximately 25 bp that is complementary to the mRNA of interest, a short linker region, and one half of a split 'PreAmp' binding site. The successful hybridization of both members of a Z-probe pair side-by-side on a target mRNA allows the binding of a PreAmp molecule (Wang et al., 2012). Bound PreAmp molecules allow the binding of Amp molecules, which in turn allow binding of label molecules, which either catalyze the deposition of chromogen (e.g., diaminobenzidene [DAB] stain) or are directly detected by fluorescence microscopy (e.g., fluorescein). The signal detected by microscopy is thus highly amplified (Wang et al., 2012). In fact, the results of RNAscope ISH appear punctate, unlike conventional ISH staining. A direct correlation between mRNA number per cell (obtained by QuantiGene 2.0 assays [Affymetrix, Santa Clara, California, USA]) and the number of dots per cell observed by RNAscope indicates that these spots are most likely single mRNA molecules (Wang et al., 2012).

The RNAscope method is largely being developed and marketed for use in human and animal clinical tissues. To determine whether this method could also be used to localize mRNA molecules in plant tissue sections, two genes were chosen for a proof-of-concept experiment whose expression patterns in maize leaf had already been reported in the literature. Probes were designed for phosphoenolpyruvate carboxylase (*PEPC*), a well characterized C_4 photosynthetic enzyme, which helps concentrate carbon dioxide into 'carbon-rich' molecules for transport to the bundle sheath cells (Kausch et al., 2001), and phosphoenolpyruvate carboxykinase (*PEPCK*), also a well characterized C_4 photosynthetic enzyme, which releases CO_2 from 'concentrated' carbon compounds in the bundle sheath cells, thus making CO_2 molecules available for the primary carbon fixation reactions catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO; Suzuki and Burnell, 2003).

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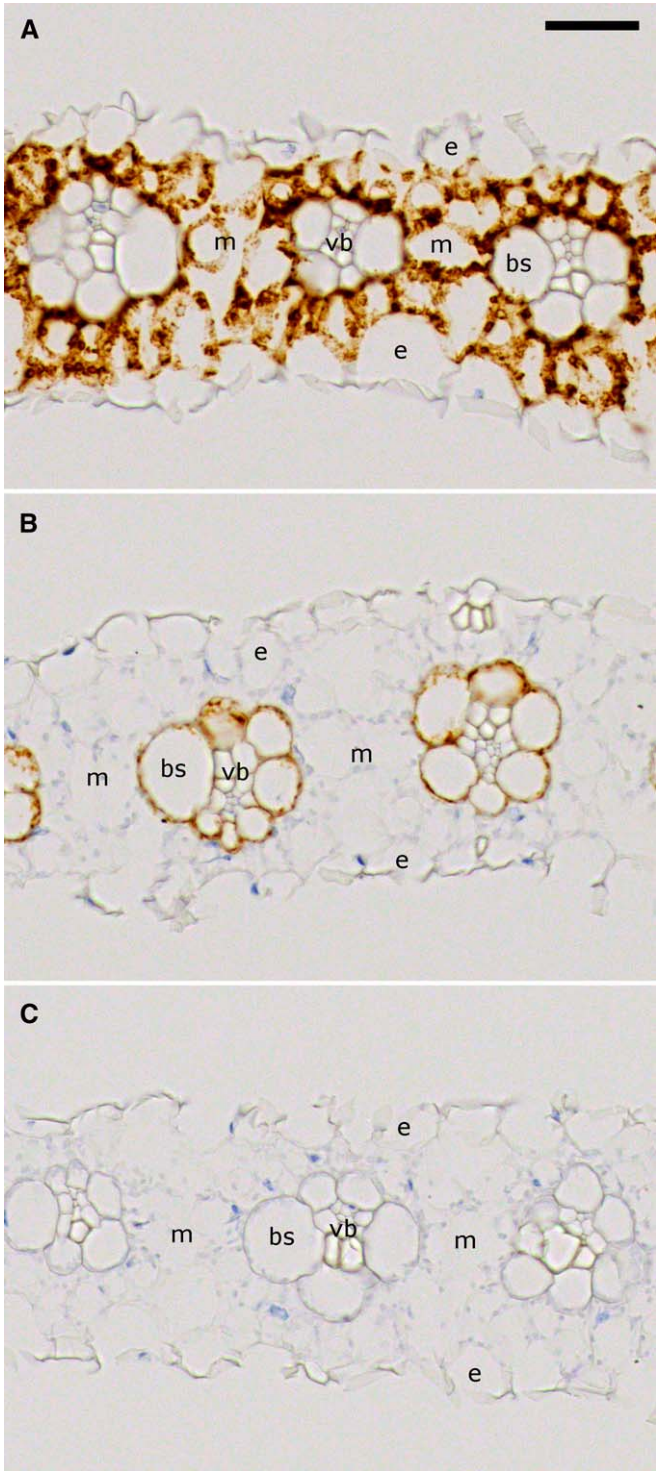


Fig. 1. Cross sections of maize leaf following RNAscope ISH using diaminobenzidene (DAB) stain as the chromogen and counterstained with hematoxylin. (A) A leaf section probed for *PEPC* mRNA showing abundant signal specifically localized to the mesophyll cells (m) of the leaf. In some regions of the section, possibly where the cell was sectioned obliquely, the brown DAB signal can be seen to be composed of a large number of individual spots or punctae. These spots are thought to represent single mRNA molecules. (B) A leaf section probed for *PEPCK* mRNA showing specific localization of these mRNA to the bundle sheath cells (bs), which surround and enclose the vascular cells in maize. Here again, the punctate DAB signal likely indicates single mRNA molecules. (C) A leaf section probed with an irrelevant probe (a bacterial gene, *dapB*), demonstrating the very low background (absence of brown DAB stain) seen with this technique. bs = bundle sheath cells; e = epidermal cells, m = mesophyll cells, vb = vascular bundle. Scale bar = 50 μm.

METHODS AND RESULTS

Fixation, infiltration, and embedding of plant material for FFPE sections—Maize plants were grown in a greenhouse under standard growth conditions to the V2–V3 stage. Small pieces (2–3 mm) of maize leaf were harvested from approximately the midblade area, placed into fixative solution (4% formaldehyde in 10 mM phosphate-buffered saline [PBS] with 1 : 10,000 Silwet L-77 [Lehle Seeds, Round Rock, Texas, USA]), and vacuumed several times until tissues sank, and then fixed overnight at 4°C. Fixed leaf tissue was then embedded in paraffin using a microwave-assisted (PELCO BioWave Pro, Ted Pella Inc., Redding, California, USA) infiltration technique to generate formalin-fixed paraffin-embedded (FFPE) plant tissue specimens (Schichnes et al., 2005; see Appendix 1).

Slide preparation for Advanced Cell Diagnostics RNAscope ISH procedure—Seven-micron-thick FFPE cross sections of maize leaf were cut using a Microm HM 315 microtome (Microm International, Walldorf, Germany) and applied to Fisherbrand Superfrost Plus slides (Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA). Sections were allowed to dry on slides for 3 h and then baked in an Agilent G2545A Hybridization Oven (Sheldon Manufacturing, Cornelius, Oregon, USA) for 1 h at 60°C. After baking, slides were dewaxed in xylene and dehydrated in EtOH in preparation for pretreatment steps according to a modified slide preparation protocol (see Appendix 2).

After the dewaxing and pretreatment steps have been completed, slides may be processed for RNAscope ISH according to the Manual ACD protocol (RNAscope 2.0 HD Detection Kit [Red] User Manual, http://www.acdbio.com/support/technical-doc) or semiautomated ACD RNAscope VS Assay (RNAscope VS Reagent Kit [Brown] User Manual for use with Ventana Discovery Ultra System [Ventana Medical Systems, Tuscon, Arizona, USA]; http://www.acdbio.com/support/technical-doc). Barcode labels for the semiautomated RNAscope VS Assay were generated using an E-Bar II barcode slide label system (Ventana Medical Systems). Ventana VSS software (version 12.1 with custom VSS mRNA DAB Improvements) was used to program the semiautomated RNAscope VS Assay according to manufacturer's instructions (RNAscope VS Reagent Kit [Brown] User Manual for use with Ventana Discovery Ultra System; http://www.acdbio.com/support/technical-doc). Both the 'manual' and 'semi-automated' RNAscope protocols were tested to validate this ISH method for FFPE plant tissues.

Probes were produced using the custom probe design service offered by ACD. Maize accessions GRMZM2G083841_T02 (*PEPC*) and GRMZM2G001696_T01 (*PEPCK*), obtained from http://www.maizesequence.org, were submitted to ACD for probe design and synthesis.

Slight modifications were made to the original ACD protocol for preparing and mounting slides for the semiautomated RNAscope VS Assay (see Appendix 2 for details). Slides were imaged on a Leica DM5000 upright microscope (Leica Microsystems, Buffalo Grove, Illinois, USA) with a Leica DFC310FX camera and Leica Application Suite software (version 4.0). Figure panels were created with GNU Image Manipulation Program (GIMP, version 2.8; http://www.gimp.org/).

Results—Using the RNAscope ISH method, mRNA coding for *PEPC* was detected primarily in the mesophyll cells of the maize leaf, as expected (Fig. 1A). *PEPCK* mRNA molecules were localized almost exclusively to the maize leaf bundle sheath cells, also as expected (Fig. 1B). The localization of the individual spots to the periphery of the cells is due to the fact that these cells have very large central vacuoles that force their cytoplasm into a thin rim around the inside of the cell wall, and is therefore indicative of subcellular localization by this technique. A negative control was performed with a probe directed to an irrelevant bacterial gene, *dapB*, and no signal was observed (Fig. 1C), demonstrating the very low background signal generated by this technique.

The successful localization of these two genes in precisely the locations reported in the literature, coupled with the extremely low background observed on these tissues, demonstrates the applicability of the RNAscope ISH method to FFPE plant tissue sections. These results are far superior to those obtained

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with conventional, radio-labeled probes (Martineau and Taylor, 1986; Langdale et al., 1988; Malone et al., 2007). In addition to localization information, the punctate nature of the detected mRNA signals also allows for direct quantification of the expression of genes by RNAscope ISH through simple counting of the resultant detection spots (Wang et al., 2012).

We began our evaluation of the RNAscope method on plant tissues using the standard protocol from the ACD website (www.acdbio.com) with the probes shown in Fig. 1 (*PEPC*, *PEPCK*, and *dapB*). For the manual method, it is important to note that the HybEZ hybridization oven (ACD) was used for all hybridization and amplification steps. The manual method involves multiple rounds of insertion and removal of the slide staining rack from the oven to apply and remove each of the hybridization and amplification reagents, so the use of this low-internal-volume oven, which comes back to temperature more quickly than a standard-size oven, could be an important factor. Although the manual method yielded excellent results, the RNAscope method has also been formulated for use with the Ventana Discovery Ultra automated slide staining system (Ventana Medical Systems), so we also tested the automated version of this method. The use of this automated staining platform to localize mRNA expression patterns allows for a more flexible workflow for the rather lengthy 8–10 h RNAscope staining protocol, as well as increased repeatability by reducing human-induced variability. However, for the genes and probes tested in this study, the automated RNAscope method yielded labeling equivalent to the manual method, so the automated staining equipment is not required for the successful use of RNAscope.

CONCLUSIONS

The RNAscope ISH method yields highly sensitive and specific mRNA expression localizations on conventional FFPE sections of plant tissues. Also, the short turnaround time for the design and synthesis of the probes allows for completion of in situ hybridization experiments in just a few weeks after initial conception. This method has also shown consistently low background levels, which is a common problem with conventional ISH methods. Finally, the ability of this method to be automated by the Ventana Discovery Ultra slide stainer allows for maximal repeatability and minimal human-induced error in these lengthy protocols.

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Appendix 1. Preparation of FFPE plant tissues using a microwave-assisted method for paraffin infiltration of plant tissues. Adapted by A. J. Bowling and H. E. Pence from Ruzin (1999) and Schichnes et al. (2005).

Day 1

1. Prepare fresh 4% formaldehyde solution for tissue specimen dissection and fixation (refer to Table A1 for reagent and equipment details):
   a. In a fume hood, combine 30 mL of 10 mM phosphate-buffered saline (PBS), 4 µL Silwet L-77, and 1 ampoule of 10 × 10 mL 16% formaldehyde in a small beaker and mix well with stir bar.
   b. Add 2–5 mL of 4% fixative solution to each scintillation vial; reserve a small amount of solution for dissecting tissues.
2. Cut tissue into 3–4-mm pieces in a nonreactive dish with a small amount of prepared fixative solution.
3. Place dissected samples in scintillation vials containing fixative solution, and vacuum several times to remove air from tissues. Tissue(s) will sink when sufficiently vacuumed. Fix tissues overnight at 4°C.

Table A1. Reagent and equipment list for preparation of FFPE plant tissues.[a]

Reagents and equipment	Supplier/manufacturer	Catalog/part no.
Scintillation vials	Thermo Fisher Scientific	03-337-7
Stir plate	VWR International	310
Stir bar	Thermo Fisher Scientific	22-067-181
Plastic beaker (50 mL)	Thermo Fisher Scientific	14-955-111A
Glass beaker (50 mL)	Thermo Fisher Scientific	S30731
Graduated plastic pipettes	Electron Microscopy Sciences	70962-9
PELCO BioWave Pro	Ted Pella Inc.	36500
SteadyTemp water bath	Ted Pella Inc.	50061
Casting molds	Electron Microscopy Sciences	62352-15
100% EtOH	Thermo Fisher Scientific	BP2818-500
100% IPA	Thermo Fisher Scientific	A416-1
Xylenes	Thermo Fisher Scientific	X5-1
DI H_2O	—	—
10 mM PBS	Sigma-Aldrich Co.	P3818-10PAK
Silwet L-77	Thermo Fisher Scientific	NC0138454
16% Formaldehyde	Ted Pella Inc.	18505
Melted paraffin (Paraplast Plus)	Ted Pella Inc.	18393

Note: DI H_2O = deionized water; EtOH = ethanol; IPA = isopropanol; PBS = phosphate-buffered saline.

[a]Manufacturer location information not provided in the text is as follows: VWR International, Radnor, Pennsylvania, USA; Electron Microscopy Sciences, Hatfield, Pennsylvania, USA; Sigma-Aldrich Co., St. Louis, Missouri, USA.

Day 2

1. Prepare water bath and temperature probe for PELCO BioWave Pro (Ted Pella Inc., Redding, California, USA) according to manufacturer's instructions.
2. Follow steps in Table A2 to complete microwave-assisted paraffin infiltration for plant tissues.
   a. Use 8 mL total volume in vials for all steps.
   b. Adjust height of the water bath to be roughly equal to the solution level in the vial.
   c. Use melted paraffin for all wax steps (Table A2, steps 10–14).
   d. Prepare solutions at least one step ahead and put these prepared solutions in water bath with sample vial.
   e. Keep a pipette and plastic beaker in a 60°C oven for removing waste paraffin during steps 10–14.
   f. In a 50-mL glass beaker, dip ~20 mL of water from SteadyTemp water bath (Ted Pella Inc.) to insulate vial during waste paraffin removal (steps 10–14). This prevents solidification of melted paraffin.
3. Cast blocks after microwave infiltration. Allow blocks to cool to room temperature and transfer to 4°C overnight prior to sectioning.

Table A2. Steps for PELCO BioWave Pro–assisted infiltration of paraffin for plant tissues.

Step	Fluid	Time (min)	SteadyTemp (°C)	Watts	TempProbe (°C)
1	25% EtOH	5	10	500	77
2	50% EtOH	5	10	500	77
3	75% EtOH	5	10	500	77
4	95% EtOH	5	10	500	77
5	100% EtOH	5	10	500	77
6	100% EtOH	5	10	500	77
7	50% EtOH : 50% IPA	5	10	500	77
8	100% IPA	5	10	500	77
9	Heavy mineral oil	10	60	500	77
10	Wax 1	30	60	500	77
11	Wax 2	30	60	500	77
12	Wax 3	30	60	500	77
13	Wax 4	30	60	500	77
14	Wax 5	30	60	500	77

Note: EtOH = ethanol; IPA = isopropanol.

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Appendix 2. This protocol describes a modified slide preparation protocol for the semiautomated RNAscope VS Assay using the reagents and equipment listed in Table A3. Protocol modified from the RNAscope VS Reagent Kit (Brown) User Manual for use with Ventana Discovery Ultra System (http://www.acdbio.com/support/technical-doc).

Day 1

1. Cut 7-µm-thick FFPE sections and mount on Fisherbrand Superfrost Plus slides (Thermo Fisher Scientific). Allow sections to dry for at least 3 h.
2. Bake slides for 1 h in a 60°C oven. Remove slides and begin semiautomated RNAscope VS Assay (proceed to steps 3–8) or store slides in a covered box overnight and begin assay on Day 2 if desired.

Day 2

3. Prepare ACD Pretreat 2 solution:
   a. Mix one bottle of ACD 10× Pretreat 2 solution (70 mL) with 630 mL deionized H_2O in a 1-L glass beaker; this will make 700 mL of 1× Pretreat 2 solution.
   b. Cover beaker with foil and heat on hot plate until 1× Pretreat 2 solution reaches 95–104°C; monitor temperature closely with a thermometer.
4. While Pretreat 2 solution is heating, dewax and dehydrate slides:
   a. Submerge slides in first Coplin jar of 100% xylene for 5 min at room temperature; agitate periodically.
   b. Repeat step 4a two more times with second and third Coplin jars. Slides will have a total of 15 min dewaxing in 100% xylene.
   c. Submerge slides in first Coplin jar of 100% EtOH for 1 min at room temperature; agitate periodically.
   d. Repeat step 4c with the second Coplin jar of 100% EtOH.
   e. Allow slides to air dry in fume hood; apply barcode labels to slides.
5. Place labeled slides in Tissue-Tek rack and submerge in beaker containing heated Pretreat 2 solution. Allow slides to heat for 15 min, maintaining solution temperature at 95–104°C.
6. Proceed with the semiautomated RNAscope VS Assay protocol as described in the RNAscope VS Reagent Kit (Brown) User Manual for use with Ventana Discovery Ultra System (http://www.acdbio.com/support/technical-doc).
7. RNAscope VS Assay runs for approximately 8 h on the Ventana Discovery Ultra platform.
8. To mount slides after assay:
   a. Remove slides from drawers; carefully tap off excess solution.
   b. Place slides in Tissue-Tek rack and submerge in hot soapy water (2% Dawn dish detergent); agitate slides 8–10 times.
   c. Transfer Tissue-Tek rack to staining dish with deionized H_2O; agitate rack 3–5 times.
   d. Repeat step 8c with fresh deionized H_2O.
   e. Transfer slides to Coplin jar with 75% EtOH for 2 min; agitate periodically.
   f. Transfer slides to first Coplin jar with 100% EtOH for 2 min; agitate periodically.
   g. Repeat step 8f with second Coplin jar of 100% EtOH.
   h. Transfer slides to first Coplin jar with 100% xylene for 1 min; agitate periodically.
   i. Repeat step 8h with second Coplin jar of 100% xylene.
   j. Mount slides out of xylene with EcoMount or Poly-Mount Xylene; allow to cure in a fume hood overnight prior to imaging.

Table A3. Reagent and equipment list for preparation of slides for the semiautomated RNAscope VS Assay.[a]

Reagents and equipment	Supplier/manufacturer	Catalog/part no.
Microtome	Microm International	HM315
Hybridization oven	Agilent (Sheldon Manufacturing)	G2545A
Superfrost Plus slides	Thermo Fisher Scientific	12-550-15
Tissue-Tek Vertical 24 slide rack	Thermo Fisher Scientific	NC9837976
Tissue-Tek staining dish	Thermo Fisher Scientific	22-149-429
Forceps	Ted Pella Inc.	523
Glass beaker (1 L)	Thermo Fisher Scientific	NC0005402
Hot plate	Corning Inc.	PC-220
Aluminum foil	Ted Pella Inc.	43-100
Thermometer	Thermo Fisher Scientific	13-201-414
ACD Pretreat 2 (10× concentrate)	Advanced Cell Diagnostics	320600
DI H_2O	—	—
100% EtOH	Thermo Fisher Scientific	BP2818-100
Xylenes	Thermo Fisher Scientific	X5-1
5 Coplin jars	Ted Pella Inc.	428-3
EcoMount or Poly-Mount Xylene	Biocare Medical or Polysciences Inc.	EM897L or 24176-120
Cover glass 24 × 30–50 mm	Thermo Fisher Scientific	12-553-462, 460, 471

Note: DI H_2O = deionized water; EtOH = ethanol.

[a] Manufacturer location information not provided in the text is as follows: Corning Inc., Tewksbury, Massachusetts, USA; Biocare Medical, Concord, California, USA; Polysciences Inc., Warrington, Pennsylvania, USA.

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