sRNA-FISH: versatile fluorescent in situ detection of small RNAs in plants

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SUMMARY
Localization of mRNA and small RNAs (sRNAs) is important for understanding their function. Fluorescent in situ hybridization (FISH) has been used extensively in animal systems to study the localization and expression of sRNAs. However, current methods for fluorescent in situ detection of sRNA in plant tissues are less developed. Here we report a protocol (sRNA-FISH) for efficient fluorescent detection of sRNAs in plants. This protocol is suitable for application in diverse plant species and tissue types. The use of locked nucleic acid probes and antibodies conjugated with different fluorophores allows the detection of two sRNAs in the same sample. Using this method, we have successfully detected the co-localization of miR2275 and a 24-nucleotide phased small interfering RNA in maize anther tapetal and archesporial cells. We describe how to overcome the common problem of the wide range of autofluorescence in embedded plant tissue using linear spectral unmixing on a laser scanning confocal microscope. For highly autofluorescent samples, we show that multi-photon fluorescence excitation microscopy can be used to separate the target sRNA-FISH signal from background autofluorescence. In contrast to colorimetric in situ hybridization, sRNA-FISH signals can be imaged using super-resolution microscopy to examine the subcellular localization of sRNAs. We detected maize miR2275 by super-resolution structured illumination microscopy and direct stochastic optical reconstruction microscopy. In this study, we describe how we overcame the challenges of adapting FISH for imaging in plant tissue and provide a step-by-step sRNA-FISH protocol for studying sRNAs at the cellular and even subcellular level.

Keywords: Zea mays, Litchi chinensis, Oryza sativa, fluorescent in situ hybridization, microRNA, sRNA, multi-photon microscopy, immunofluorescence, technical advance, LNA probes.

INTRODUCTION
Ribonucleic acid is information, and localization of this information is critical for its function. Plants and animals have several pathways leading to the production of developmental and functionally important small RNAs (sRNAs) [21–24 nucleotides (nt) in size]. These sRNAs can act in a homology-dependent manner to guide transcriptional and post-transcriptional silencing (Reinhart et al., 2002; Xie et al., 2010; Berezhikov, 2011). Recent studies have demonstrated cross-kingdom and host-pathogen movement of microRNAs (miRNAs) that can regulate gene expression in vitro (Shahid et al., 2018). The subcellular localizations of the components of miRNA and short interfering RNA (siRNA) pathways have been described, and include Cajal bodies (CBs) (Fang and Spector, 2007; Pontes and Pikaard, 2008), dicing bodies (D-bodies) (Fang and Spector, 2007), processing bodies (P-bodies) (Pontes and Pikaard, 2008) and the more recently described membrane-bound poly-somes (Li et al., 2016). In animal cells, siRNA and miRNA dicing occurs in the nucleus and cytosol (Fang and Spector, 2007). In plant cells, miRNA processing occurs in the nucleus via DICER-LIKE 1 (DCL1) and other miRNA-processing proteins (Fang and Spector, 2007).
In the past, precise subcellular and even cellular imaging of the production of sRNAs was challenging in plants. The
two main methods are green fluorescent protein (GFP)-based sensors and colorimetric in situ hybridization. Small RNAs have been detected indirectly using a GFP-based sRNA sensor that detects the silencing of GFP by a sRNA such as miR156 (Nodine and Bartel, 2010). This method has been used in grafting experiments to detect the shoot-to-root movement of mobile sRNAs that direct transcriptional gene silencing (Melnik et al., 2011). Similar GFP-based sRNA sensors have been used in Caenorhabditis elegans to investigate the systemic spreading of gene silencing between tissues (Winston et al., 2002). These sensors reveal a loss of signal, but lack quantitative and precise spatial resolution. Methods for localization of mRNA by in situ hybridization are routine, but they lack the sensitivity to enable these methods to be directly adapted for most sRNAs. A major advancement has occurred with the advent of locked nucleic acid (LNA) oligonucleotide probes that have improved affinity, sensitivity and specificity (Vester and Wengel, 2004). LNA have been used in combination with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) for colorimetric localization of sRNAs in the maize shoot apex (Javelle and Timmermans, 2012), mouse brain (Bak et al., 2008), Drosophila embryos (Rozhkov et al., 2011) and, by us, in maize anthers (Zhai et al., 2015). Using this method, hydrolysis of BCIP by phosphatase produces a blue-colored precipitate at the site of enzymatic activity that is easy to see using transmitted light microscopy but cannot be detected by microscopes that require fluorescence (Trinh le et al., 2007). Whole plant- or tissue-level in situ hybridizations were made possible by using 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC), a chemical that crosslinks the 5′ end of sRNAs to protein, with a miRNA preserved through the washes and tissue clearing (Ghosh Dastidar et al., 2016). However, these non-fluorescent, colorimetric methods are not easily modified for multiplexed detection, and they are poorly suited for the generation of three-dimensional (3D) images.

Fluorescent in situ hybridization (FISH) has been used extensively in animal systems to study the localization and expression of sRNA. Obernosterer et al. (2007) used a Fast Red substrate instead of NBT/BCIP for detection of miRNAs in mouse brain. Dual-target fluorescence in situ hybridization assays were also used for detecting pathogens in cell cultures (Shah et al., 2017), as well as the specific chromosomal location related to invasive breast tumors (Walker et al., 2013). In higher eukaryotes, the fine structure of RNA-processing bodies, including P-bodies, D-bodies and CBs, have been challenging to observe using conventional microscopy because of their small size (300–500 nm in diameter), which is close to the diffraction limit of light (Mito et al., 2016). Meanwhile, super-resolution microscopy, including structured illumination microscopy (SIM) and stochastic optical reconstruction microscopy (STORM), images beyond the diffraction limit of light. Markaki et al. (2013) combined 3D SIM and FISH on cultured mammalian cells to analyze the spatial relations and substructures of nuclear targets. Stochastic optical reconstruction microscopy was used in combination with RNA-FISH to determine the relative distance between nascent transcripts, with a precision of a few tens of nanometers (Larkin and Cook, 2016). The sensitivity and specificity of these methods inspired us to develop a similar technique for plant tissues, in combination with LNA-locked in situ hybridization for detection of sRNA targets.

The sRNA-FISH we developed is based on the colorimetric sRNA in situ method (Javelle and Timmermans, 2012). We show that sRNA-FISH can be used to assay two sRNA targets in the same sample. Furthermore, plants exhibit strong autofluorescence, which often confounds FISH imaging. We show that, for many samples, laser scanning confocal microscopy (LSCM) can be used to spectrally separate autofluorescence from the RNA-FISH signal. For highly autofluorescent samples, multi-photon fluorescence excitation alters the autofluorescence spectrum so that it can be easily separated from the sRNA-FISH signal, leading to an increase in the signal-to-noise ratio and specificity. With little adaptation, the sRNA-FISH protocol can be used with super-resolution SIM (SR-SIM) and STORM for subcellular localization of sRNAs.

RESULTS AND DISCUSSION

Development of fluorescent in situ methods to analyze sRNA localization

Maize (Zea mays) anthers from early development stages were selected as the model system for developing a sRNA-FISH method. Our method is a modification of the Javelle and Timmermans protocol (Javelle and Timmermans, 2012) for colorimetric sRNA in situ hybridization in plant tissues. Figure 1 provides an overview of the workflow. A detailed step-by-step protocol and reagents list can be found in Protocol S1 in the online Supporting Information.

Samples, in this case maize flower buds, were dissected and immediately fixed to avoid degradation of sRNA. The fixative was in PHEM buffer (5 mM HEPES, 60 mM PIPES, 10 mM EGTA, 2 mM MgCl2) because it provides better preservation of the overall cell structure than PBS buffer (see Experimental Procedures and Protocol S1). The PHEM buffer was originally developed to preserve the microtubule distribution and pericentriolar material (Houlston et al., 1987). Another critical step is vacuum infiltration; this helps the fixative penetrate into tissues with air spaces. For hybridization we used LNA-modified probes with a digoxigenin (DIG) N-hydroxysuccinimide (NHS) ester group added to the 5′ or 3′ end of the probes. Locked nucleic acid probes have high sensitivity and specificity; they are suitable for analysis of short RNA and DNA targets.
After pre-hybridization, probe hybridization and post-hybridization washing and blocking, probes can either be detected using the colorimetric method or by immunofluorescence methods. Up to the post-hybridization step, our protocol can be used for either fluorescent or colorimetric detection. Both methods have their advantages, and we compare them below. For immunofluorescence detection, samples are incubated with primary antibodies to the probes and secondary antibodies with chosen fluorophores. We tested two primary-secondary antibody combinations. Mouse anti-DIG IgG primary with goat anti-mouse conjugated with Alexa Fluor® 488 (AF488) resulted in background signal in anther cells with either the specific probe or a scramble control. In contrast, the combination of sheep anti-DIG Fab fragment primary and donkey anti-sheep IgG AF568 secondary led to successful in situ hybridization in maize tissues. After antibody washing, samples were mounted in SlowFade® Gold or Diamond Antifade Mountant and stored at 4°C until imaging.

Linear spectral unmixing of autofluorescence for specific detection of the sRNA-FISH signal

Due to the autofluorescence that occurs in paraffin-embedded plant tissue, all confocal and multi-photon microscope images needed to be spectrally unmixed. Laser-scanning confocal microscopes with spectral imaging are becoming increasingly more common (Zimmermann et al., 2014), and nearly all microscopes from the major manufacturers can be equipped with this capability. Linear spectral unmixing of fluorophores from autofluorescence is essential for the sRNA-FISH protocol described here because nearly all paraffin-embedded plant tissues have a significant amount of autofluorescence that can vary based on species or tissue type. Two important controls are needed to conduct linear spectral unmixing. First, a sample with no secondary antibodies, which have the fluorescence dye conjugate, must be examined with different laser excitations to determine the autofluorescence spectra of the paraffin-embedded sample section. The spectra must be saved and will be used later for unmixing. This step is critical, as it provides the spectral information for choosing fluorophores that have the smallest amount of overlap with the autofluorescence. Second, once a fluorophore has been chosen a solution of the pure secondary antibody conjugated to the fluorophore should be mixed with the mounting medium and imaged to obtain pure spectra of the fluorophore. The spectra of the autofluorescence and the pure fluorophore fluorescence can then be used to unmix the tissue autofluorescence from the specific sRNA-FISH signal. All the laser scanning confocal and multi-photon microscopy images shown here were acquired using spectral detection followed by linear spectral unmixing.

Comparison of sRNA-FISH and colorimetric detection of the same targets

In order to examine the specificity and sensitivity of sRNA-FISH we compared our method with the colorimetric method. miR2275 and miR2118 are abundant miRNAs that

Figure 1. Workflow of small RNA fluorescent in situ hybridization. Starting with sample preparation and probe design, tissues were fixed, embedded, sectioned and adhered to glass slides. Critical steps include determining sample autofluorescence and choosing antibodies with the right fluorophore combinations. After imaging, linear spectral unmixing is necessary for precise localization of small RNAs. DAPI, 4',6-diamidino-2-phenylindole.

(Vester and Wengel, 2004). After pre-hybridization, probe hybridization and post-hybridization washing and blocking, probes can either be detected using the colorimetric method or by immunofluorescence methods. Up to the post-hybridization step, our protocol can be used for either fluorescent or colorimetric detection. Both methods have their advantages, and we compare them below. For immunofluorescence detection, samples are incubated with primary antibodies to the probes and secondary antibodies with chosen fluorophores. We tested two primary-secondary antibody combinations. Mouse anti-DIG IgG primary with goat anti-mouse conjugated with Alexa Fluor® 488 (AF488) resulted in background signal in anther cells with either the specific probe or a scramble control. In contrast, the combination of sheep anti-DIG Fab fragment primary and donkey anti-sheep IgG AF568 secondary led to successful in situ hybridization in maize tissues. After antibody washing, samples were mounted in SlowFade® Gold or Diamond Antifade Mountant and stored at 4°C until imaging.

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are important for anther development, with a maximum observed abundance in fertile maize anthers of 12 100 transcripts per 10 million reads (TP10M) for miR2275 in 1-mm anthers, and 11 433 TP10M for miR2118 in 0.4-mm anthers (Zhai et al., 2015). Both miRNAs have known localization patterns, with miR2275 being abundant in maize anthers of length 1.0–2.0 mm and localized to the tapetal layer and archesporial cells (Zhai et al., 2015). miR2118 is enriched in pre-meiotic maize anthers and is mainly localized to the epidermal cell layer (Zhai et al., 2015). We designed probes for both miR2275 and miR2118 (probe sequences are listed in Table 1). The sRNA-FISH and colorimetric methods both showed the same localization of miR2275 and miR2118 for 1.5- and 0.4-mm maize anthers, respectively (Figure 2). For miR2275, we observed a strong signal (red in the fluorescent method and dark in the colorimetric method) in the tapetal layer and archesporial cells using both methods. For miR2118, we observed a signal mainly in the epidermal layer of early stage anthers, also using both methods. The specific signals in the different locations for miR2275 and miR2118 probes were detectable compared with no signal for the scrambled probe control. The traditional colorimetric in situ method provides greater signal amplification, which can be seen for miR2118 detection. It is the best method when maximum sensitivity is required for tissue-level expression studies of one sRNA target. However, FISH is a powerful strategy for overcoming the limitations of colorimetric-based detection schemes (Clay and Ramakrishnan, 2005; Barroso-Chinea et al., 2007). Specifically, our sRNA-FISH method is a better choice when more than one sRNA target needs to be detected simultaneously and when cellular and subcellular localization of sRNAs is required. Below we describe how our basic sRNA-FISH method can be applied for the detection of two sRNA targets or super-resolution localization of sRNAs.

Applying sRNA-FISH to detect and co-localize two sRNAs in the same sample

To extend our in situ method from one sRNA target to two sRNA targets, we included LNA probes conjugated with 3'-carboxyfluorescein (FAM™), which is an NHS ester-modified single isomer derivative of fluorescein. The FAM fluorescence was not detected directly (it was quenched by the hybridization protocol), but rather was amplified and detected with anti-fluorescein rabbit antibody and donkey Fab anti-rabbit IgG secondary conjugated with AF568. To test this method, we designed two LNA probes to miR2275 and its 24-nt phased siRNA (phasiRNA). The probe for miR2275 was conjugated with 3'-DIG and the probe for 24-nt phasiRNA was conjugated to 3' 6-FAM. They were hybridized to 1.5-mm maize anther sections because the sRNA targets are both present and abundant at this stage (Zhai et al., 2015). Using both DIG- and fluorescein-labeled probes in the same hybridization experiment, we were able to detect specific fluorescence for both miR2275 and the 24-nt phasiRNA compared with the scrambled LNA probe controls (Figure 3). The 24-nt phasiRNA (Figure 3, cyan) and miR2275 (Figure 3, magenta) co-localized to the tapetal layer and archesporial cells.

Use of multi-photon excitation to mitigate plant autofluorescence

Above, we have shown that our sRNA-FISH method can be used with LSCM for both single- and dual-target sRNA detection in maize anthers. However, LSCM uses one-photon excitation that can exhibit much higher levels of autofluorescence in certain types of plant tissues and species. Imaging is dependent on having a high signal-to-background ratio, and these high levels of autofluorescence can prevent the detection of weaker signals below the autofluorescence level. For example, we were unable to use one-photon detection of sRNA-FISH in litchi (Litchi chinensis Sonn.) anthers because the autofluorescence with either 561- or 633-nm laser excitation was similar to the emission spectrum of AF568 or AF647 fluorophores (Figure 4). This was evident in both the lambda-coded spectral image that depicts true color and the graphed spectra of the autofluorescence and AF dyes (Figure 4a). In comparison, using multi-photon excitation, we were able to overcome this autofluorescence issue. Imaging with

Table 1 Information on probes used in this study

<table>
<thead>
<tr>
<th>miRNA</th>
<th>miRNA abundance (TP10M)</th>
<th>Probe sequence</th>
<th>Probe Tm (°C)</th>
<th>Hybridization temperature (°C)</th>
<th>Probe concentration (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>zma-miR2118</td>
<td>4064</td>
<td>/5DigN/TAGGAATGGGAGGCATCGGGAA</td>
<td>86</td>
<td>53.3</td>
<td>250</td>
</tr>
<tr>
<td>24-phasiRNA</td>
<td>16 318</td>
<td>GGCCAAGGTCCGGTCCAACAACT/36-FAM/</td>
<td>87</td>
<td>53.3</td>
<td>250</td>
</tr>
<tr>
<td>zma-miR2275</td>
<td>12 100</td>
<td>GTGTCAGTGGCCTTATACCTCT/3DigN/</td>
<td>88</td>
<td>53.3</td>
<td>250</td>
</tr>
<tr>
<td>24-phasiRNA for litchi</td>
<td>837</td>
<td>TCTCATGTTTCTCATAGGTCT/3DigN/</td>
<td>83</td>
<td>53.3</td>
<td>250</td>
</tr>
<tr>
<td>Osa-miR1874</td>
<td>59 000</td>
<td>ATCGGTTACCCCTCCATCCATA/3DigN/</td>
<td>86</td>
<td>53.3</td>
<td>100</td>
</tr>
<tr>
<td>Scrambled control</td>
<td>NA</td>
<td>/5DigN/TTGTAACACGTCTATACGCCC/</td>
<td>87</td>
<td>53.3</td>
<td>250</td>
</tr>
<tr>
<td>Scrambled Control</td>
<td>NA</td>
<td>TGTACACGCTTATACGCCC/36-FAM/</td>
<td>87</td>
<td>53.3</td>
<td>250</td>
</tr>
</tbody>
</table>

TP10M, transcripts per 10 million reads; phasiRNA, phased small interfering RNA; Tm, melting temperature; 5DigN, 5’-digoxigenin N-hydroxysuccinimide ester; 3DigN, 3’-Digoxigenin N-hydroxysuccinimide Ester; 36-FAM, 3’- 6-FAM Fluorescein.
745-nm multi-photon excitation exhibited blue-to-green autofluorescence that was well separated from the distinct orange fluorescent signal of AF568. The 745-nm multi-photon excitation also exhibited a second autofluorescence peak around 655 nm that was mainly outside the anther. The emission peak of an AF568-conjugated secondary antibody (603 nm) falls between the two autofluorescence spectra, making it possible to linearly spectrally unmix it from the two autofluorescence peaks (Figure 4). We also tested this approach in rice (Oryza sativa) endosperm, another highly autofluorescent tissue type. Exactly the same area of the endosperm section was imaged with either one-photon or multi-photon excitation (Figure S1). Small RNA-FISH of osa-miR1874 showed numerous discrete spots of signal by multi-photon excitation and many of these spots could not be spectrally unmixed from the autofluorescence using one-photon excitation. In summary, multi-photon excitation microscopy can be used to overcome the high autofluorescence levels of plant samples that cannot be imaged with one-photon LSCM.

Development of sRNA-FISH for detecting subcellular localization of sRNAs

Introducing fluorescence to in situ hybridization has enabled subcellular localization of RNAs. However, the diffraction limit of light restricts the resolution of LSCM to about 200 nm in the focal plane (x,y) and about 450 nm in the optical (z) axis, making details of the subcellular structures and RNA assemblies unresolvable (Hell, 2007; Huang et al., 2016). A multitude of super-resolution light microscopy techniques have emerged that can surpass the diffraction limit of light (Rust et al., 2006; Huang et al., 2010). Here, we examined whether two super-resolution approaches, SR-SIM and direct STORM (dSTORM), can be applied to sRNA-FISH. The SR-SIM approach utilizes moiré fringes to render otherwise unresolvable high-resolution information (Gustafsson, 2005). The high-frequency information in each SR-SIM image is used to double the resolution to about 100 nm (Kner et al., 2009). Probes against miR2275 were used to detect miR2275 expression in maize anthers at the pre-meiotic stage. The result was very...
Figure 3. Dual-target small RNA fluorescent in situ hybridization (sRNA-FISH) for maize anthers.
(a) Both zma-miR2275 (detected in the AF633 channel; magenta) and the 24-nucleotide (nt) phased small interfering RNA (phasiRNA) (detected in the AF568 channel; cyan) were detected in the tapetal layer and archesporial cells by sRNA-FISH. Each image was collected in spectral mode using laser scanning confocal microscopy and then spectrally unmixed using Zen software. Bright-field and merged images are also shown for each image. TA, tapetal layer; AR, archesporial cells. Scale bars = 20 μm for all images.
(b) Quantification of the AF633 and AF568 signal intensity in dual-target sRNA-FISH and controls (significance level: *<0.05; **<0.01).
similar to fluorescent and colorimetric detection of miR2275 expression in maize anthers at the same stage – that is using SR-SIM; miR2275 is also mainly localized to the tapetal layer and archesporial cells. We also detected miR2275 in secondary parietal cells, which later give rise to the middle layer and tapetal cells (Kelliher et al., 2014) (Figure 5). The original STORM method used two dyes and required the dyes to be conjugated to the same antibody. In contrast, dSTORM uses only one dye and commercially available secondary antibodies are readily available (Heilemann et al., 2008). Both methods require the molecules to be driven into a dark state and the detection of single molecules that stochastically enter the fluorescent state; a Gaussian fit of fluorescence of spatially separated single molecules can be used to determine their location to approximately 20 nm (Hell, 2007). That single-molecule detection is also the key to capturing a dSTORM image without linear spectral unmixing. A single AF647 dye molecule emits significantly more photons than autofluorescence. Filtering based on the high photon counts of AF647 was used to minimize background autofluorescence without linear spectral unmixing. Using dSTORM (Figure 6), we were able to obtain localization of miR2275 at the subcellular level with a precision of \(<20\) nm (Table S1), although the size of the antibodies will decrease the actual resolution. With the super-resolution of dSTORM images, detailed localization of each sRNA can be achieved. In Figure 6(b), we have captured the precise localization of
Figure 5. Localization of miR2275 in pre-meiotic maize anthers using structured illumination microscopy (SIM). Top left panel: laser wide-field images show that miR2275 is detected in the archesporial cells and secondary parietal cells; the latter give rise to the middle layer and tapetum. Bottom left panel: detection of miR2275 using super-resolution structured illumination. miR2275 is localized to archesporial and secondary parietal cells. Right panels are images of the scrambled probe control. AR, archesporial cells; SPC, secondary parietal cells; EN, endothecium; EPI, epidermis. Scale bar = 20 μm for all images.

Figure 6. Localization of miR2275 in pre-meiotic maize anthers using direct stochastic optical reconstruction microscopy. (a) miR2275 was detected in the tapetal layer and archesporial cells. In comparison, the scrambled probe yields a very low signal. (b) Higher-magnification images of boxes 1 and 2 showing localization around the nucleus (Nu) and in the cytosol (Cy). TA, tapetal layer; AR, archesporial cells.
miR2275 around and in the nucleus and in the cytosol. These localization events as well as brightness and the precision radius were documented with exact positions as \( x \) and \( y \) coordinates (Table S1).

Overall, FISH in plant tissue is challenging, and in this work we present a sRNA-FISH protocol that takes advantage of many of the features of fluorescence including multiple probes to detect multiple targets in the same sample and high-resolution localization at the cellular and subcellular level. Another challenge when working with plant tissue is the amount of autofluorescence in many tissues. Multi-photon excitation with a 745-nm laser results in two distinct autofluorescence peaks in most of the plants we tested, including rice and litchi; this gives us a wide spectral window for selecting fluorophores. Single-photon excitation can be used for tissues that exhibit less autofluorescence, such as maize anthers, and LSCM systems are more readily accessible. Here we demonstrate that our sRNA-FISH method can be applied to a wide variety of plant tissue types and species. It complements the traditional colorimetric in situ hybridization method that has much higher signal amplification. Our detailed step-by-step protocol provided in the file Protocol S1 is identical for colorimetric in situ hybridization and sRNA-FISH until the step of antibody incubation (step 25), and it can be used to compare the two methods. We have found that the colorimetric in situ hybridization method should be applied when the aim is maximum sensitivity at the tissue-to-organ level. However, these non-fluorescent colorimetric methods are not easily modifiable for multiplexed detection. In contrast, the sRNA-FISH method can be easily adapted to dual-detection of two RNA targets. The fact that sRNA-FISH requires antibody amplification complicates its use for absolute copy number quantification of sRNAs. A bioinformatically designed, oligonucleotide-based technology called OligoPAINT enables single-molecule super-resolution imaging of chromosomes without antibody amplification (Beliveau et al., 2015). Adaptation of a similar method to sRNAs should yield a fine-resolution imaging method with a precise number of fluorophores or binding sites.

Finally, the cellular mechanism of RNA transport and its role in sRNA biogenesis is still unclear. Nucleolus-associated CBs in plants have been implicated as sites of biogenesis of siRNA and miRNA (Pontes and Pikaard, 2008). DCL1 and DCL3-Ago4 (Argonaute protein 4) siRNA processing centers can be located in highly dynamic CBs (Pontes and Pikaard, 2008). However, DCL1 and the double-stranded RNA-binding domain-like superfamily protein 1 (HYL1) can function in a non-CB-dependent manner when localized to D-bodies, suggesting that D-bodies have a role in processing of primary miRNA and miRNA biogenesis (Fang and Spector, 2007). For both animals and plants, P-bodies contain all the components for miRNA-directed cleavage: Argonaute proteins and miRNAs forming the RNA-induced silencing complex and untranslated mRNA (Liu et al., 2005). Cellular fractionation and molecular analysis of phasiRNA biogenesis showed that phasiRNAs are generated from miRNA-targeted transcripts on membrane-bound polysomes, and the miRNAs are recruited to the membrane in an AGO1-dependent manner (Li et al., 2016).

In the future, sRNA-FISH may be combined with other, compatible fluorescence-based methods to detect other RNA and protein targets. The ability to localize sRNAs, potentially other RNA targets including mRNA transcription and sRNA precursors, together with proteinaceous biogenesis components all at a subcellular level may yield breakthroughs in our understanding of RNAs.

**EXPERIMENTAL PROCEDURES**

**Plant material**

Maize samples were kindly provided by the Walbot Lab at Stanford University, California. Anthers of the W23 inbred line were grown in Stanford under greenhouse conditions. Anthers were dissected and measured using a micrometer as previously described (Kelliher and Walbot, 2011). Rice samples were provided by Dr Yuanlong Liu from the Donald Danforth Plant Science Center. Litchi samples were kindly provided by Dr Rui Xia from the South China Agricultural University (China). *Arabidopsis* (Col-0) plants were grown on 0.5 MS/0.8% agar (MS agar) plates in controlled-environment chambers under 16-h light/8-h dark at a temperature of 23°C.

**Probe design**

The LNA-modified oligonucleotide probes were synthesized by Exiqon (Exiqon Qiagen, https://www.qiagen.com/us/shop/genes-and-pathways/exiqon-lna-products/). The webpage for this probe design can be found at https://www.qiagen.com/us/shop/pcr/primersets/custom-lna-oligonucleotides/#orderinginformation. The input requires specific sRNA sequences and species. The output contains probe sequences, melting temperature \( (T_m) \) and molecular weight. The LNA position information will not be released. Scrambled control probes were directly ordered from Exiqon (cat. no. YD00690004). All probe sequences are listed in Table 1.

**Sample preparation**

A detailed protocol of sample preparation, including fixation and embedding, can be found in Protocol S1. Briefly, anthers were dissected and fixed in a 20-ml glass vial using 4% paraformaldehyde in 1× PHEM buffer (5 mM HEPES, 60 mM PIPES, 10 mM EGTA, 2 mM MgCl\(_2\) pH 7). Fixation was done three times in a vacuum chamber at 0.08 MPa for 15 min each time. After fixation, samples were sent for paraffin embedding at the histology lab at Nemours/Alfred I. duPont Hospital for Children (Wilmington, DE, USA). Samples were sectioned using a paraffin microtome and dried on Fisherbrand™ Tissue Path SuperFrost™ Plus Gold Slides (cat. no. 15-188-48, Thermo Fisher Scientific, http://www.thermofisher.com/).

**Fluorescent in situ hybridization**

Fluorescent in situ hybridization was modified from the protocol described by Javelle and Timmermans (2012) by replacing the antibody with a primary anti-DIG Fab fragment (cat. no. 11214667001, Sigma-Aldrich, http://www.sigmaaldrich.com/) and
secondary donkey anti-sheep IgG (H+L) AF647, AF568 or AF633 (cat. nos A-21448, A-21099 and A-21100, Thermo Fisher Scientific). For two-color in situ hybridization, a 6-FAM™ labeled probe was amplified with anti-fluorescein (Abbcam ab19491, https://www.abcam.com/) from rabbit and donkey Fab'2 anti-rabbit IgG H&L (Alexa Fluor® 568) (Abbcam ab 175694). For a detailed protocol, please refer to Protocol S1. Briefly, samples were de-paraffinized using Histo-Clear (cat. no. 50-899-90147, Fisher Scientific) and rehydrated by going through an ethanol series of 95%, 80%, 70%, 50%, 30%, 10% (vol/vol) (for 30 sec each) and water (1 min) at room temperature (20°C). After protease (PS147, Sigma) digestion (20 min at 37°C), samples were treated with 0.2% glycine (G3889, Sigma-Aldrich) for 2 min followed by a TEA treatment (Triethanolamine, Sigma-Aldrich, 90279; HCl and acetic anhydride, Sigma-Aldrich, A6404). After two washes in 1× PBS buffer, samples were dehydrated and then hybridized with probes overnight at 53.3°C. Ten milliliters of hybridization buffer contains 875 μl of nuclelease-free H₂O, 1.25 ml of in situ hybridization salts, 5 ml of deionized formamide, 2.5 ml of 50% (w/v) dextran sulfate, 250 μl of 50× Denhard's solution and 125 μl of 100 mg ml⁻¹ tRNA. Hybridized slides were then washed twice using 0.2 x saline-sodium citrate buffer, blocked in 1× blocking buffer (1% blocking reagent in 1× TRIS-buffered saline (TBS) buffer), and 1× washing buffer (1% w/v BSA; A7906, Sigma-Aldrich) and 0.3% Triton X-100 in 1× TBS buffer for 1 h each. Samples were then incubated with primary antibody overnight at 4°C followed by four washes in 1× washing buffer (15 min each wash). Samples were then incubated with a secondary antibody overnight at 4°C followed by four washes in 1× washing buffer (15 min each wash). After a final wash in 1× TBS buffer, the samples were mounted using SlowFade™ Gold Antifade Mountant (S36936, ThermoFisher Scientific) or SlowFade™ Diamond Antifade Mountant (S36867, ThermoFisher Scientific). The other antibody combination we tested was mouse anti-DIG IgG primary (cat. no. 11333062910, Sigma-Aldrich) with goat anti-mouse conjugated with AF488 secondary (A11017, Thermo Fisher Scientific), but this resulted in non-specific background plant tissue.

Image acquisition

Bright-field images of colorimetric in situ hybridization were obtained on a Zeiss Axioplan 2 using an AxioCam MR color camera. Spectral imaging was conducted on a Carl Zeiss LSM 880 laser scanning microscope capable of both LSCM and multi-photon microscopy. The Zen software (Carl Zeiss). The images were processed using the same parameters, as follows: discard overlapping molecules; peak mask ware (Carl Zeiss). The images were obtained for each of the five grid orientations, the collection was shifted to five different rotations. For each field of view, a five-slice z-stack was taken and then maximum-intensity images were generated using the stacks with Zen software (Carl Zeiss). A 642-nm laser (10%) was used to excite the AF633, and a 405-nm laser (20%) was used to excite 4′,6-diamidino-2-phenylindole (DAPI). After image subsets were obtained for each of the five grid orientations, the collection was analyzed in Zen (Carl Zeiss). The images were processed using the same parameters, which are as follows: theoretical point spread function; baseline cut display; SR-frequency weighting (1); noise filter (-1); sectioning (100%, 83%, 83%).

dSTORM imaging

The dSTORM images were taken using a Zeiss Elyra PS.1 super-resolution microscope with a Plan-Apochromat 100×/1.46 oil objective with 642-nm (100%) and 405-nm (10%) laser excitation. Anther samples were sectioned and dried on medium-density wide spectral band coverglass (600 ± 100 nm fiducials, density no. (100 μm)² 51–150) (Hestzig LLC, http://hestzig.com/) coated with poly-L-lysine (Sigma). After sRNA fluorescent in situ hybridization, the sample was then mounted in a dSTORM imaging buffer. The dSTORM buffer was made by mixing three buffers immediately before use: solution A (containing 30 nM TRIS/Ci pH 8.5, 1 mm EDTA, 6.25 μM glucose oxidase, and 2.5 μM catalase for oxygen scavenging); solution B containing (250 mM cysteamine-HCl, pH 3); and solution C (containing 250 mM glucose in water). Samples were sealed in a magnetic CF chamber (Chamlide, http://www.chamlide.com/) during imaging. Images were taken with an exposure time of 100 ms, with an EMCCD gain of 30 (40 000 frames in total). Images were aligned using a fiducial-based algorithm. After image subsets were obtained for each image, the raw data were analyzed in Zen software (Carl Zeiss). The images were processed using the same parameters, as follows: discard overlapping molecules; peak mask ware (Carl Zeiss). The images were obtained for each of the five grid orientations, the collection was shifted to five different rotations. For each field of view, a five-slice z-stack was taken and then maximum-intensity images were generated using the stacks with Zen software (Carl Zeiss). A 642-nm laser (10%) was used to excite the AF633, and a 405-nm laser (20%) was used to excite 4′,6-diamidino-2-phenylindole (DAPI). After image subsets were obtained for each of the five grid orientations, the collection was analyzed in Zen (Carl Zeiss). The images were processed using the same parameters, which are as follows: theoretical point spread function; baseline cut display; SR-frequency weighting (1); noise filter (-1); sectioning (100%, 83%, 83%).

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY INFORMATION

Additional Supporting Information may be found in the online version of this article.


Table S1. Coordinates of miR2275 localization detected by small RNA fluorescent in situ hybridization and direct stochastic optical reconstruction microscopy imaging.

Figure S1. Comparison of one-photon and multi-photon excitation imaging resolution in rice endosperm amyloplast.
REFERENCES


Bereczik, E. (2011) Evolution of microRNA diversity and regulation in ani-


Clay, H. and Ramakrishnan, L. (2005) Multiplex fluorescence in situ hybridiza-


Fang, Y. and Spector, D.L. (2007) Identification of nuclear dicing bodies con-


Houliston, E., Pickering, S.J. and Maro, B. (1987) Redistribution of micro-


Javelle, M. and Timmermans, M.C.P. (2010) Breaking the diffraction bar-


Li, S., Le, B., Ma, X. et al. (2016) Biogenesis of phased siRNAs on mem-