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POINT-OF-VIEW

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Gaining insight into plant gene transcription using smFISH

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ABSTRACT
Single molecule RNA fluorescent *in situ* hybridization (smFISH) enables gene transcription to be assessed at the cellular level. In this point of view article, we describe our recent smFISH research in the model plant *Arabidopsis thaliana* and discuss how this technique could further knowledge of plant gene transcription in the future.

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Introduction

Plant protein research has benefited for many years from a variety of cell biology techniques, however plant RNA studies have been limited by a lack of methods that enable subcellular resolution imaging. Traditionally, *in situ* hybridization (ISH) has been used to render plant RNA visible. Radio-labeled nucleotide probes designed to bind specific targets through Watson–Crick base pairing were first used for this purpose.[1] Later, Digoxigenin (DIG) probes (detectable by anti-DIG antbodies conjugated to alkaline phosphatase), became more popular as they generate longer lasting signals and are easier to handle than radioactive probes.[2] This chromogenic method of RNA labelling has since been adapted for use in many plant species and continues to be used routinely to determine endogenous gene expression across a range of plant tissues.[3]

ISH relies on non-linear signal amplification for RNA visualization, therefore it cannot provide quantitative transcript data. In addition, ISH enzymatic reactions generate molecules that diffuse away from target RNA, so information relating to subcellular localization is lost. Both of these limitations were first overcome outside of the plant research field by the Singer lab.[4] They developed a single molecule RNA FISH (smFISH) method where transcripts are detected using 50 base pair fluorescently labelled DNA probes. This technique enabled individual RNA molecules to be imaged and quantified for the first time. A commercial version of this method has since been developed where 48 fluorescently labeled, 20 base pair probes can be ordered to label RNA molecules of interest[5] (LGC Biosearch Technologies). This shorter probe length, together with a minimum of ~25 binding events required for transcript visualization, provides a high level of both sensitivity and specificity. While smFISH has been extensively used in many other model systems, this method has only recently been optimized for plant research.[6] Unlike transgenic approaches that have been developed to visualize individual plant RNA molecules at the cellular level *in vivo* (see Tilsner 2014)[7], smFISH detects endogenous RNAs and can be readily applied to any genetic background. Moreover, the simplicity of this method makes it suitable for large-scale studies of coding and noncoding RNA with applications in developmental, cell and systems biology.[8,9] smFISH not only reveals the localization of RNA in tissues and within cells, but is now being used as a powerful technique to reveal detailed mechanistic insights into gene regulation at the cellular level. Here, we will discuss our recent smFISH work and comment on new opportunities this method brings to the field of plant transcription.

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Making individual plant RNAs visible

We adapted an existing smFISH protocol for *C. elegans*[5] for use in *Arabidopsis thaliana* root meristem cells to investigate transcriptional regulation of *Flowering Locus C* (*FLC*). This gene is a potent repressor of flowering that is epigenetically silenced following a prolonged cold treatment – known as vernalization.[10] Transcriptional shut-down of *FLC* is observed as a prelude to accumulation of repressive histone modifications that ensure stable repression of the gene. In this system, sense transcription significantly reduces after around two weeks of cold and this coincides with the upregulation of a set of alternatively spliced antisense *FLC* transcripts known as *COOLAIR*.[11] These long non-coding transcripts are required for coordinated switching from active to repressed chromatin states at *FLC*.[12] Knowledge of how transcription and non-coding RNA contribute to *FLC* silencing had been largely based on results from bulk assays that yield data averaged across many cells. However, it had been demonstrated that each allele alters chromatin states independently[13] and epigenetic switching of *FLC* is cell autonomous.[14] These insights prompted us to develop an smFISH method for plants to investigate sense and antisense *FLC* transcription, as well as *FLC* mRNA abundance, at the cellular level.

Plant fluorescence microscopy is complicated by endogenous autofluorescence, so we developed our smFISH method for *Arabidopsis thaliana* root cells where this is minimal. First, we validated our method by imaging mRNA from the housekeeping gene *PP2A* and developed an automated image analysis pipeline to quantify mRNA and determine cell-to-cell variation.[6] Next, we used probes specifically targeted to *FLC* exons to visualize mRNA and quantify per cell reductions during a four week vernalization experiment[15] (see Table 1). This time-course dataset provided quantitative validation for our method as it revealed *FLC* mRNA fold reductions that closely matched qPCR results after two and four weeks of cold exposure. By designing probes to exclusively bind the first intron of *FLC* RNA, we were able to detect sites of active transcription[16] (see Table 1). In contrast to a gradual reduction of *FLC* mRNAs, we observed a more rapid reduction in active transcription. Rare intronic signals after 2-weeks of cold, combined with the presence of cytoplasmic mRNA after 3 weeks provided additional evidence of an extended *FLC* mRNA half-life in the cold. [12]But as we observed a homogeneous distribution of cytoplasmic mRNA throughout vernalization, we could confirm this was not due to aggregation of transcripts within intracellular storage compartments.[17]

Next, we used smFISH to investigate *COOLAIR* transcription. In agreement with low levels of *COOLAIR* expression detected by qPCR, our intronic probe set (designed to detect antisense nascent transcripts) revealed very few cells transcribing *FLC* in the antisense orientation prior to cold. During two weeks of cold exposure we found that the number of cells expressing *COOLAIR* increased rapidly. In addition, the *COOLAIR* signal intensities increased significantly. We combined smFISH with immunofluorescence to rule out that these *COOLAIR* accumulations occurred within Cajal bodies. By incorporating DNA FISH into our protocol, we were able to confirm that these large *COOLAIR* foci co-localized with *FLC*, leading us to speculate that they contribute towards *FLC* transcriptional shutdown in a *cis*-manner.[15]

By establishing this method, we finally had the tools to investigate the relationship between sense and antisense transcription at the single cell and single locus level. By combining sense and antisense probe sets, we confirmed that while *FLC* sense and *COOLAIR* can co-occur in the same cell, they are never transcribed from the same allele at a given time. Further analysis showed significant anti-correlation between sense and

Table 1. Design considerations for smFISH probe sets.

Probe Set Target	RNA labelled	Location	Additional Information
Intron Probes	Nascent RNA	Nucleus	Chromosomal location of gene (only applicable where the gene is spliced in a co-transcriptional manner)
Exon Probes	mRNA & Nascent RNA	Nucleus & Cytoplasm	Produces quantifiable spots that indicate the number of transcripts present in each cell.
Probe set that labels both exons and introns	mRNA & Nascent RNA	Nucleus & Cytoplasm	Relative intensity measurements can be used to infer transcriptional bursts. Can also be used to determine the number of transcripts. Higher intensity signals may be visible in the nucleus that represent active sites of transcription.

antisense expression at all time-points, at individual loci. We could rule out transcriptional interference as a cause for this mutual exclusivity, since *COOLAIR* does not run through the *FLC* promoter and collisions were not detected by smFISH. Previous qPCR analysis of *FLC* and its antisense *COOLAIR* in different genotypes had previously indicated a positive correlation.[11] However, this apparent contradiction between smFISH and the whole cell population data can be reconciled – while sense and antisense are mutually exclusive at the level of transcription, they are both similarly influenced by *trans*-factors and the local chromatin environment that determine the general state of the locus.

In a more recent study, we combined single-molecule RNA FISH and computational modelling to investigate the kinetics of *FLC* transcription and the relationship between *FLC* and *COOLAIR* transcription dynamics.[18] We revealed that cell-to-cell variability in transcript abundance is much broader than described with a single Poisson process, but could almost entirely be explained by the fact that transcript abundance scales linearly with cell size. After correcting for this deterministic source and errors in cell size measurements we concluded that the remaining variability is only minimally stochastic, and can be explained by a single Poisson process. Using pulse-chase experiments, in combination with the transcription elongation inhibitor Actinomycin D, we further showed that the degradation rate of transcripts in single cells remains constant and is independent of cell size. We concluded that it must be the transcription rate itself that scales with cell size. Moreover, *FLC* transcription was shown to be largely continuous and non-bursty (i.e. there was an absence of stochastic activation/ inactivation of transcription leading to discontinuous production of mRNA). Using different probe sets for *FLC* (exons, full-length intron and different regions of the intron) we further estimated rates of transcription initiation, elongation, transcript processing, and release from the locus. These measurements indicated that transcription initiation scales with cell size, and that transcript processing and release from the locus are relatively slow. Finally, we showed that in cells where *COOLAIR* is transcribed, the transcription of *COOLAIR* scales with cell size, and that this abolishes the cell size scaling of *FLC* as expected given their mutually exclusive relationship.[15]

More recently, smFISH has also been used to investigate an intriguing role for transcriptional regulation in nutrient uptake. Boron is essential for cell wall integrity, but is cytotoxic at high concentrations. It is imported via root NIP5;1 transporters through outer cell layers (epidermis, cortex and endodermis) into the vasculature where it is distributed throughout the plant. Mathematical modelling was used to investigate the importance of transporter regulation rates in this system after rapid responses were observed following boron concentration changes made in the lab. In the model, when the parameter for *NIP5;1* regulation was reduced to a rate lower than determined *in vivo*, this was found to trigger oscillatory waves of intracellular boron concentrations that propagated in an opposing direction to nutrient flow. Next, we used smFISH to gain a better insight into rapid NIP5;1 regulation. Our images revealed that under normal boron conditions, (where there is a minimal requirement for import), *PP2A* transcripts were dispersed homogenously throughout the cell, however probes targeted to *NIP5;1* exons were mostly observed as either one or two bright spots restricted within nuclei. By combining *NIP5;1* exon and intron probe sets with different dyes, consistent co-localization indicated that nascent *NIP5;1* RNA was being turned over at sites of transcription. These smFISH results are indicative of a system where protein expression is not simply limited by switching transcription off. Rather, transcription remains switched on and degradation machinery limits mRNA production and export to the cytoplasm. This provides the plant with a sensitive and highly dynamic system that can respond rapidly to intracellular and external environmental conditions to facilitate smooth, polarized nutrient flow.[19]

Pushing the boundaries: Limitations of plant smFISH

A major drawback for the use of smFISH in plant research is the challenge posed by autofluorescence, especially in green tissues. Presently, we believe that our smFISH protocol for Arabidopsis roots can be easily adapted to suit other plant species. But ultimately, highly specific signal amplification, combined with clearing and sectioning will be required to extend smFISH analysis across more plant tissues.

We have also attempted to amplify signals to overcome autofluorescence using both commercial

(Affymetrix) and bespoke branched probe sets (similar to those reported by Sinnamon and Czaplinski[20]). Both of these approaches have been reported to generate brighter spots per RNA molecule than smFISH.[8,20] They use initial primary sets that partially bind to target RNA with overhanging sequences that are partially complimentary to the first section of the secondary probe set. The sets are hybridized sequentially to create branched oligo "trees" that are labelled by a fluorescent tertiary probe set. For our three RNA targets (*mVENUS*, *ACTIN* and *PP2A*), both commercial and bespoke probe sets increased signal intensity sufficiently to enable confocal imaging. But this benefit was outweighed by unacceptable false-negative and false-positive detection rates. Although target specificity and sensitivity are likely to differ between targets, our preliminary data indicate that considerable optimisation will be required before branched FISH probes can be considered a robust alternative for quantifying RNA at the cellular level in plants.

What next? – New possibilities for plant transcription research

Transcription is essential to all life and full understanding of this complex process ultimately requires the quantification of the different kinetic parameters influencing the transcription cycle. Measurements of gene expression in single cells by smFISH in yeast, Drosophila and mammals have revealed surprising cell-to-cell variability otherwise hidden in bulk measurements.[21–23] Our work has provided a glimpse into the extent of cell-to-cell variability in plant gene expression. We believe that given their sessile nature and susceptibility to environmental conditions, large-scale smFISH studies in plants could provide further important insights into environmental interactions and transcription kinetics. In all systems tested so far where stochasticity in gene expression has been reported, it still remains unclear to what extent this can be attributed to intrinsic stochasticity or to extrinsic variation due to cell-cycle, cell size or other features. By combining smFISH with computational modelling its possible for these important questions to be addressed in plants in the future.

smFISH is also a powerful tool to study different aspects of mRNA life-cycle. For instance, in the case of *FLC,* we have shown that transcription inhibitors can be used effectively to determine mRNA half-life.[18] In addition, intronic probes that detect nascent RNAs can be used to determine transcriptional activity of individual loci. Furthermore, the ability to visualize RNA at sub-cellular resolution can reveal nuclear or cytoplasmic localization. Both of these forms of retention have been shown to have consequences for gene expression.[8,19,24]

In many cases measurements from smFISH labelling has been shown to provide valuable information regarding the nature of transcription itself. For instance, the presence of large cell-to-cell variability in mRNAs in addition to the presence of intense foci in the nucleus can be interpreted in most cases as a sign of transcriptional bursting. Indeed, in mammalian liver cells, smFISH approaches applied together with computational models have revealed that gene expression in this tissue consists mostly of transcriptional bursts.[25] Finally, high-throughput versions of RNA FISH have been developed to generate reproducible, quantitative transcriptomic data with high sensitivity.[8,26] Similar approaches in plants will make smFISH not only a powerful approach to study gene expression at the cellular level, but also a useful tool for high-content screening assays.

Conclusions

The ability to measure gene expression parameters, namely transcription and degradation rates, and burst fractions in single cells opens new avenues to explore the physical properties of transcription in plants. Following on from these advances there will be a wide range of research areas where smFISH could provide useful insights. Undoubtedly, these novel possibilities will improve understanding of plant transcription and RNA biology in the future.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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