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Corresponding Author:	David Stanek Ustav molekularni genetiky Akademie Ved Ceske Republiky Prague, - CZECH REPUBLIC
Corresponding Author's Institution:	Ustav molekularni genetiky Akademie Ved Ceske Republiky
Corresponding Author E-Mail:	stanek@img.cas.cz
Order of Authors:	Adriana Roithova
	David Stanek
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TITLE:

Analysis of Spliceosomal snRNA Localization in Human Hela Cells Using Microinjection

AUTHORS & AFFILIATIONS:

Adriana Roithová[1,2], David Staněk[1]

[1]Laboratory of RNA Biology, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic

[2]Department of Genetics and Microbiology, Faculty of Science, Charles University, Prague, Czech Republic

Corresponding Author:

David Staněk

Email Address: stanek@img.cas.cz

Email Address of Co-author:

Adriana Roithová (roithova@img.cas.cz)

KEYWORDS:

microinjection, snRNA, HeLa cells, Cajal body, nucleus, cytoplasm

SHORT ABSTRACT:

Biogenesis of spliceosomal snRNAs is a complex process involving various cellular compartments. Here, we employed microinjection of fluorescently labelled snRNAs in order to monitor their transport inside the cell.

LONG ABSTRACT:

Biogenesis of spliceosomal snRNAs is a complex process involving both nuclear and cytoplasmic phases and the last step occurs in a nuclear compartment called the Cajal body. However, sequences that direct snRNA localization into this subnuclear structure have not been known until recently. To determine sequences important for accumulation of snRNAs in Cajal bodies, we employed microinjection of fluorescently labelled snRNAs followed by their localization inside cells. First, we prepared snRNA deletion mutants, synthesized DNA templates for in vitro transcription and transcribed snRNAs in the presence of UTP coupled with Alexa488. Labelled snRNAs were mixed with 70 kDa-Dextran conjugated with TRITC, and microinjected to the nucleus or the cytoplasm of human HeLa cells. Cells were incubated for 1 h and fixed and the Cajal body marker coilin was visualized by indirect immunofluorescence, while snRNAs and dextran, which serves as a marker of nuclear or cytoplasmic injection, were observed directly using a fluorescence microscope. This method allows for efficient and rapid testing of how various sequences influence RNA localization inside cells. Here, we show the importance of the Sm-binding sequence for efficient localization of snRNAs into the Cajal body.

INTRODUCTION:

RNA splicing is one of the crucial steps in gene expression, which is catalyzed by a large ribonucleoprotein complex called the spliceosome. In total, more than 150 proteins and 5 small

nuclear RNAs (snRNAs) are integrated into the spliceosome at different stages of the splicing pathway. U1, U2, U4, U5 and U6 snRNAs are participating in splicing of major GU-AG introns. These snRNAs join the spliceosome as pre-formed small nuclear ribonucleoprotein particles (snRNPs) that contain snRNA, seven Sm proteins associated with snRNA (or Like-Sm proteins, which associate with the U6 snRNA) and 1-12 proteins specific for each snRNP.

Assembly of snRNPs involves cytoplasmic and nuclear stages. Newly transcribed snRNA is exported to the cytoplasm where it acquires a ring assembled from seven Sm proteins. The Sm ring subsequently serves as a signal for snRNA re-import back to the nucleus. Defective snRNAs that fail to associate with Sm proteins are retained in the cytoplasm[1]. Newly imported snRNPs first appear in the Cajal body where they meet snRNP-specific proteins and finish their maturation (reviewed in[2,3]). We recently showed that inhibition of final maturation steps results in sequestration of immature snRNPs in Cajal bodies[4,5]. We proposed a model where the final snRNP maturation is under quality control that monitors addition of snRNP-specific proteins and the formation of active snRNPs. However, molecular details of how cells distinguish between correctly assembled mature and aberrant immature particles remain elusive.

To determine snRNA sequences that are essential for targeting and accumulation of snRNAs in nuclear Cajal bodies, we decided to employ microinjection of fluorescently labelled snRNAs. Microinjection was a method of choice because: 1) it does not require an additional sequence tag to distinguish synthetic snRNAs form their endogenous counterparts which is especially important for short RNAs with little space for insertion of extra tag sequence; 2) it allows analysis of sequences that are important for biogenesis. For example, the Sm sequence is essential for Sm ring assembly and re-import into the nucleus[6]. When snRNAs are expressed in the cell, snRNAs lacking the Sm sequence are degraded in the cytoplasm and do not reach the nucleus and Cajal bodies[7]. However, snRNAs without the Sm sequence can be directly microinjected into the nucleus and thus a potential role of the Sm sequence in Cajal body localization assayed.

Here, we describe in detail a microinjection method that we applied to determine snRNA sequences necessary to target snRNAs into the Cajal body[5]. We showed that Sm and SMN binding sites are together necessary and sufficient to localize not only snRNAs but various short non-coding RNAs into the Cajal body. Based on microinjection as well as other evidence, we proposed that the Sm ring assembled on the Sm binding site is the Cajal body localization signal.

PROTOCOL:

1. Preparation of snRNAs for microinjection

1.1. Prepare a DNA template containing the full-length or truncated/mutated version of snRNA by PCR using a following PCR setup: 98 °C for 60 s, 98 °C for 15 s, 68 °C for 30 s, 72 °C for 1 min, 98 °C for 15 s for 35x, and 72 °C for 5 min.

1.2. The forward primer must contain a promoter sequence for in vitro transcription just upstream of the first transcribed nucleotide. Amplify U2 snRNA sequence using the forward

primer containing the T7 promoter and the reverse primer, which ends with the last transcribed nucleotide (see **Table of Materials** for details).

1.3. Synthesize snRNA using a kit for short RNA synthesis (see **Table of Materials** for details) containing T7 RNA polymerase. Add 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.8 mM UTP, 0.2 mM UTP-Alexa488, 1.8 mM trimethylguanosine cap analogue ($m_3^{2,2,7}$G(5')ppp(5')G), 1 µL of an RNA inhibitor and 500-700 ng of the DNA template to the reaction mixture and incubate at 37 ˚C overnight.

1.4. Add 1 µL (2U) of DNase into the reaction and incubate for additional 15 min at 37 ˚C.

1.5. Isolate snRNA by acidic phenol/chloroform extraction. Remove the top water phase and add 1/10 of the original volume of 3 M sodium acetate (pH = 5.2), 3 µL of glycogen for better pellet visualization and 2.5 volumes of 100% ethanol. Incubate for 1 h at -80 ˚C, centrifuge for 10 min at 14,000 x *g* and 4 °C.

1.6. Wash the pellet with 70% ethanol and dissolve in 12 µL of nuclease-free water. The usual RNA yield was around 600 ng/µL. Store at -80 °C.

1.7. Monitor the integrity of in vitro transcribed snRNAs by agarose gel electrophoreses.

1.8. Before microinjection, dilute RNA to the final concentration 200 ng/µL in water containing 10 µg/µL dextran-TRITC 70-kDa.

2. Cells

2.1. Before the use, treat the coverslips with 1 M HCl for 1 h, wash thoroughly in distilled water and store in 100% ethanol. Acidic treatment promotes cells adhesion to prevent cell peeling during or after injection. For easier identification of injected cells, use a coverslip with a grid.

2.2. Seed HeLa or other adherent cells 1 day before microinjection on 12 mm coverslips (no. 1 or no. 1.5) to reach 50% confluency at the time of microinjection.

3. Injection

NOTE: HeLa cells were microinjected in the Dulbecco's Modified Eagle Medium (D-MEM, 4.5 g/L D-glucose containing phenol red and antibiotics). Injection was carried out using an injector and a micromanipulator equipped with the sterile needle (see **Table of Materials** for details). The whole microscopic/micromanipulator system was pre-heated to 37 ˚C for at least 4 h to prevent fluctuation of individual parts of the microscope and the microinjector.

3.1. Put the Petri dish (30mm) containing 2 mL of culture media with the coverslip in the middle into the holder of the microscope. Load 3 µL of snRNA mixture into the needle using a microloader. Install the needle into the holder of the microinjector in 45° with respect to the

surface of the Petri dish.

3.2. To find the needle, use a 10X long distance objective. First, set the speed **COARSE** on the injector and lower the needle until it touches the culture medium. Using the microscope binoculars, find the bright spot, which is the place where the needle touches the culture medium.

3.3. Change the speed to **FINE** and further lower the needle while looking into the microscope until the tip of the needle is observed. Move the needle in the middle of visual field and switch to a 40X long distance objective.

3.4. After changing the objective, select the cell and move the needle above this cell. Set the pressures and time for the cell contact (see Tips and troubleshooting in discussion).

3.5. Move the needle down into the cell and then set the lower limit on the micromanipulator. Be careful not to move the needle below the cell.

3.6. After setting the lower limit, move the needle back above the cell and press the injection button on the joystick of the injector. The needle moves automatically inside the cell to the place where the limit is set and injects the RNA mixture.

3.7. To finish, push the button **MENU** on the injector and remove the needle from the holder. Then disconnect the tube from the injector before switching off the injector and the micromanipulator.

4. Visualization of microinjected snRNAs

4.1. After the microinjection, return the cells into a CO_2 incubator and incubate at 37 °C for 1 h.

4.2. After the incubation, rinse the cells three times with phosphate buffered saline solution (PBS) at room temperature, fix for 20 min with 4% paraformaldehyde in 0.1 M PIPES pH 6.9, and wash three times with room temperature PBS. If only snRNA localization is analyzed, briefly rinse the cells in water and mount in a mounting medium with DAPI.

4.3. In case of additional protein localization, permeabilize the cells with 0.5% Triton-X100 in PBS for 5 min at room temperature. After three rinses with PBS, incubate the cells with primary and secondary antibodies and after final washes in PBS and brief rinse in water, mount in a mounting medium with DAPI.

5. Microscopy

5.1. If not imaged immediately after mounting, store the slides at 4 °C until imaging.

5.2. Acquire images using a high-end fluorescence microscopic system equipped with an

immersion objective (60X or 100X/1.4NA).

5.3. Once microinjected cells are identified, collect a stack of 20 z-sections with 200 nm z steps per sample and subject to mathematical deconvolution. Maximal projections or individual z stacks were then presented.

5.4. To quantify the fluorescent signal, draw Region of Interest (ROI) around a Cajal body identified by coilin immunostaining. Measure the intensity of the snRNA signal in the coilin-defined ROI. Next determine the intensity of snRNA fluorescence in ROI randomly placed in the nucleoplasm and calculate the ratio of RNA signal in the Cajal body and nucleoplasm.

REPRESENTATIVE RESULTS:

To monitor snRNA localization and the role of the Sm binding site in Cajal body targeting, we prepared a DNA template containing the T7 promoter and either the full-length U2 snRNA or U2 snRNA lacking the seven nucleotides (AUUUUUG) forming the Sm binding site. snRNAs were in vitro transcribed, isolated and mixed with TRITC-coupled dextran-70kDa. We microinjected the mixture containing in vitro transcribed snRNA into the nucleus or the cytoplasm of HeLa cells.

It has been previously shown that the Sm binding site is necessary for nuclear import[6]. To test whether the Sm site is also important for Cajal body accumulation, we microinjected snRNA lacking the Sm site directly into the nucleus (**Figure 1A**). Injection into the cytoplasm served as a control (**Figure 1B**). As a positive control, we microinjected full-length snRNA (**Figure 1C**,**D**). After 60 min of incubation in a CO_2 incubator, microinjected cells were fixed and the Cajal body marker coilin was visualized by indirect immunofluorescence. The full-length snRNA accumulated in Cajal bodies after both nuclear and cytoplasmic injections. In contrast, snRNA lacking the Sm site remained in the cytoplasm after microinjection into this compartment, which is consistent with previous findings[6]. The nuclear microinjection of snRNA without the Sm site revealed that the Sm binding sequence is important for Cajal body localization. U2 snRNA lacking the Sm site remained in the nucleus but did not accumulate in Cajal bodies (**Figure 1A**).

Sometimes, the microinjection into only one cellular compartment failed and TRICT-dextran-70kDa was found in both the nucleus and the cytoplasm (**Figure 2A**). These cells were discarded from further analysis. Despite the fact that the fluorescently labelled snRNAs are stored at -80 °C before the injection, RNA degradation can occur. Degraded snRNA injected into the cytoplasm is not transported into the nucleus and stays localized in the cytoplasm (**Figure 2B**). Such snRNA should be discarded, and new snRNA should be synthesized.

FIGURE AND TABLE LEGENDS:

Figure 1. Localization of microinjected snRNAs. Alexa488-labelled U2 snRNA either lacking the Sm site (**A**,**B**) or full-length (**C**,**D**) were microinjected into the cytoplasm or the nucleus of HeLa cells. Cajal bodies (arrows) are marked by coilin immunolabeling (red); snRNAs are depicted in green. Dextran-TRITC-70kDa (yellow) was used to monitor nuclear or cytoplasmic injection; DNA was stained by DAPI (blue). Arrowheads marks cytoplasmic localization of snRNA. The scale bar represents 10 µm.

Figure 2. A potential pitfall of the microinjection approach. (**A**) Microinjection into one compartment failed and WT U2 snRNA was microinjected into both the cytoplasm and the nucleus. Note that Dextran-TRITC-70kDa (yellow) is present in both cellular compartments. (**B**) WT U2 snRNA was microinjected into the cytoplasm of HeLa cells (green) but significant amount of snRNA remained in the cytoplasm (arrowheads), which indicates partial degradation of injected snRNA and a loss of the Sm binding site. Cajal bodies (arrows) are marked by coilin immunolabeling (red). DNA was stained by DAPI (blue). The scale bar represents 10 µm.

DISCUSSION:

We employed microinjection of fluorescently labelled snRNAs to determine sequences important for snRNA localization into nuclear Cajal bodies. Due to rapid and rather simple preparation of labelled RNAs (preparation of DNA template by PCR followed by in vitro transcription) the method offers effective analysis of how various sequences contribute to RNA localization. In relatively short time, we were able to analyze ten different deletions or substitutions of the U2 snRNA (ref.[5] and data not shown). For RNAs with a complex biogenesis pathway, this approach also allows testing sequences that are essential for maturation. In the case of snRNAs, the deletion of Sm binding sequences, which are required for Sm ring assembly results in sequestration and degradation of mutated snRNAs in the cytoplasm[7]. Other advantageous include that two differently labelled RNAs can be injected simultaneously and their localization directly compared within one cell. However, as various fluorochromes might modify RNA behavior, each fluorochrome must be tested individual before double labelling experiments. RNAs of several hundred nucleotides in length have been successfully injected and their localization assayed in various model systems (reviewed in[8]). The limiting step in case of longer RNAs is usually in vitro synthesis of full-length RNA. Also, injection of RNAs with pre-assembled proteins can be applied to test the effect of individual proteins on RNP localization. In our projects, we injected snRNA associated with Sm proteins to confirm the role of the Sm ring in Cajal body localization[5]. Finally, microinjection of fluorescently labelled RNAs allows direct quantification without any additional steps, as has been previously applied for snRNAs[9].

There are also several disadvantages of the methods that should be considered before launching a microinjection project. First, despite some automatization of the microinjection process, the method still requires manual skills and experience, and researchers should consider time necessary for training. The critical parts of the protocol are the preparation of intact RNAs, microinjection and then finding injected cells in the microscope (see Tips and Troubleshooting below). Second, microinjection represents a significant stress and many cells do not survive for a longer period of time. While there are some successful attempts to follow microinjected RNAs inside cells by live-cell imaging[10], we observed significant increase of cell death when we combined microinjection with live-cell imaging using fluorescence microscopy. Third, the amount of injected RNA into human cells is very low which prevents biochemical analysis of injected RNAs. This also means that it is difficult to monitor how the incorporation of fluorescently labelled nucleotides affects RNA functions. Therefore, various ratios of labelled-NTP:NTP should be incorporated into wild-type RNA and its localization assayed to get the ideal ratio between the fluorescence signal and the functionality of labelled RNA. Also, the nature of fluorochrome

might affect RNA localization. In our hands, Alexa488-UTP worked much better than Alexa546-UTP. We have not explored these differences any further, but one reason could be a bigger size and different shape of Alexa546 in comparison to Alexa488.

Taking together, RNA microinjection is a powerful method for RNA localization but should be always combined with alternative approaches. In our case, we successfully introduced MS2-binding site into U2 snRNA, monitored its localization using MS2-YFP and confirmed some key results obtained by snRNA microinjection with the MS2 system[5].

Tips and troubleshooting

Injection pressure, compensation pressure and injection time need to be determined experimentally for each cell line. We applied injection pressure (Pi) 170 hPa and compensation pressure (Pc) 50 hPa in the case of cytoplasmic microinjection and Pi=200 hPa and Pc=80 hPa in the case of nuclear microinjection. The injection time was in both cases set to 0.2 s. You can sometimes observe a slight movement of material inside the cell, which is an indication of a successful injection. If the cell bursts, you have to decrease the injection pressure. You should also check the compensation pressure via the TRITC fluorescence channel. When you see the strong stream coming out from the tip of the needle, then decrease the compensation pressure.

Between injections, always check whether the cells are successfully injected. Identify the microinjected cells via injected Dextran-TRITC using the TRITC fluorescence channel. If you do not see any microinjected cells, first increase the injection pressure and injection time. Second, check if the needle is not plugged via fluorescence channel (TRITC). If you see Dextran-TRITC weakly streaming from the tip of the needle, then the needle is functional, and the problem is likely in the setting of the lower limit for injection. The setting of the limit is a very tricky and important step. Each cell has different shape and you will need to change the limit very often to ensure proper microinjection.

In the ideal case, one should be able to microinject ~50 cells before the tip of the needle is plugged with a cell debris. Check if fluorescence comes out of the tip regularly, and if you do not see any Dextran-TRITC streaming from the tip, then the needle is blocked. To clean it, hold the **CLEAN** button on the injector for 3 s, which increases pressure and should remove the block. If it does not help, then you could try to break off the tip of the needle by hitting the bottom of the Petri dish. However, this is a very tricky procedure, which requires a significant amount of experience and there is no guarantee of success. Therefore, for beginners, the needle exchange is advisable.

Before you exchange the needle, push the **MENU** button on the injector. Take the needle a little bit up and press **HOME** on the micromanipulator. The needle will go all the way up from the medium, but the micromanipulator remembers the original position of the needle and you do not need to find the needle again. When the needle is replaced, press the **HOME** button and the needle will go to the original position and you should be able to see the tip of the needle in the microscope. After changing the needle, you have to adjust the limit. Then, push the **MENU** button on the injector again and the needle is prepared for microinjection.

During imaging, the trickiest part is to find the microinjected cells. To locate them, use TRITC channel, where the TRITC-conjugated Dextran-70kDa is much better visible than a weak Alexa488 signal of microinjected snRNAs. The coverslips with a grid are helpful here.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

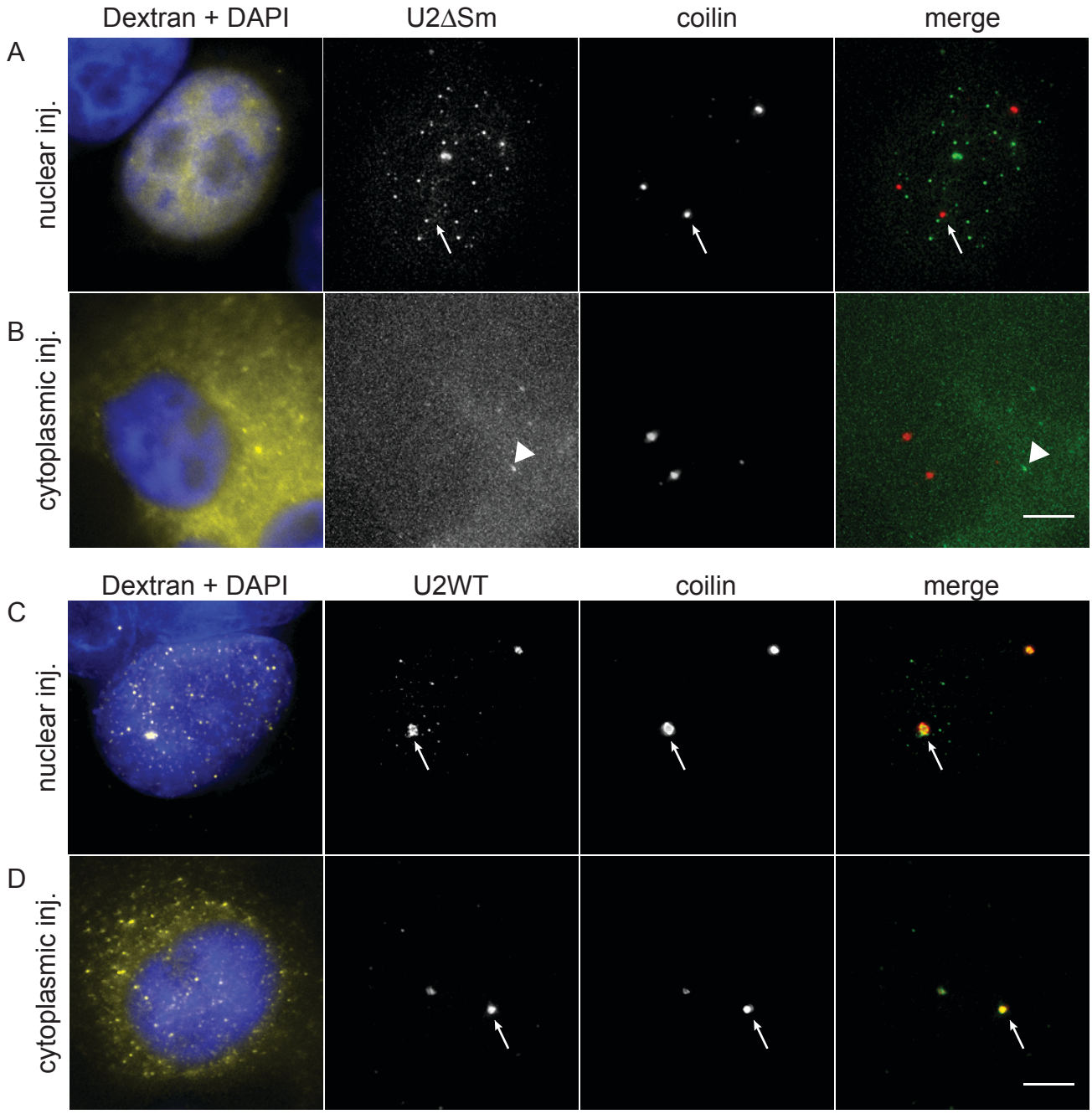

Figure 2

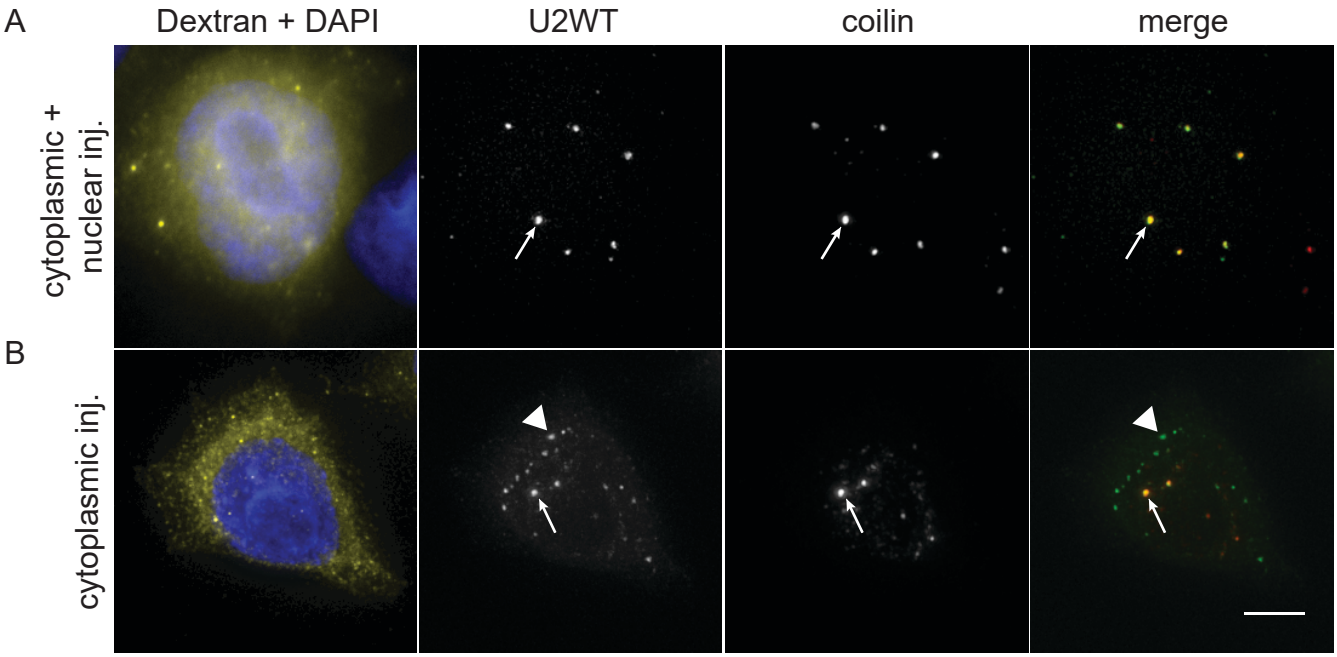

Table of Materials

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Name of Material/ Equipment	Company	Catalog #	Comments/Description
ChromaTide Alexa fluor 488-5-UTP	ThermoFisher	C11403	Stock concentration 1 mM
Dulbecco's Modified Eagle Medium - high glucose	Sigma-Aldrich	D5796	Containing 4.5 g⁄L D-glucose, Phenol red and antibiotics
FemtoJet express Injector	Eppendorf	5247000013	
Femtotips II	Eppendorf	930000043	Microinjection needle of 0.5 µm inner and 0.7 µm outer diameter
Fluoromont G with DAPI	SouthernBiotech	0100-20	
Glycogen	ThermoFisher	AM9510	Stock concentration 5 mg/mL
Gridded Glass Coverslips	Ibidi	10817	Coverslips with a grid, no direct experience with them
InjectMan NI 2 Micromanipulator	Eppendorf	5181000017	
m3-2,2,7G(5')ppp(5')G trimethyled cap analogue	Jena Bioscience	NU-853-1	Stock concentration 40 mM
MEGAshortscript T7 Transcription Kit	ThermoFisher	AM1354	
Microscope Cover Glasses 12 mm, No. 1	Paul Marienfeld GmbH	111520	For routine work
Microscope Cover Glasses 12 mm, No. 1.5	Paul Marienfeld GmbH	117520	For high resolution images
Microscope DeltaVision	GE Healthcare		For image acquisition
Microscope DMI6000	Leica		For microinjection
Paraformaldehyde 32% solution EM grade	EMS	15714	Dissolved in PIPES to the final concentration 4%
Phenol:Chloroform 5:1	Sigma-Aldrich	P1944	
Primers for U2 amplification: Forward: 5'-*TAATACGACTCACTATAGGG* ATCGCTTCTC GGCCTTTTGG, Reverse: 5´ TGGTGCACCGTTCCTGGAGGT	Sigma-Aldrich		T7 rpromoter sequence in italics
Phusion High Fidelity DNA polymerase	BioLab	M0530L	
RNasin Plus	Promega	N2615	Stock concentration 40 µM
Tetramethylrhodamine isothiocyanate Dextran 65-85 kDa	Sigma-Aldrich	T1162	Dissolved in water, stock concentration 1 mg/mL
Triton-X100	Serva	37240	Dissolved in water, stock concentration 10%

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CORRESPONDING AUTHOR

Name:	David Staněk
Department:	RNA Biology
Institution:	Institute of Molecular Genetics, CAS
Title:	Group leader
Signature:	
Date:	Feb-6-2019

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David Staněk, Ph.D.
Group-leader
Laboratory of RNA Biology

Bing Wu, Ph.D.
Review Editor
JoVE
1 Alewife Center
Cambridge
MA-02140

Manuscript: JoVE59797R1

Prague, March 7, 2019

Dear dr. Wu,

Please, find attached a revised version of our manuscript entitled "Analysis of spliceosomal snRNA localization in human HeLa cells using microinjection" for consideration for publication in *the Journal of Visualized Experiments*.

As you will see, we implemented all editorial as well as editorial suggestions. Please, find below detailed answer to individual editorial comments.

I hope that the revised version would be acceptable for publishing in *the Journal of Visualized Experiments.*

With best regards,

David Stanek

Vídeňská 1083, 142 20 Prague 4, Czech Republic | Phone: +420 296 443 118 | Fax: +420 224 310 955 | stanek@img.cas.cz | www.img.cas.cz

Response to editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done

2. Please avoid long steps (more than 4 lines).

We have split some steps of the Protocol and shorten all steps except one step to reach the 4-line-limit. The steps 1.3 exceeds the limit by less than a half of the line and their further reduction would negatively affect the comprehensibility of the Protocol. We further move primer sequences from the Step 1.1 into the Table of Materials (submitted as the file "Roithova-Table of Materials-revised-R2").

3. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done.

4. Step 1.2: Please ensure that all text is written in the imperative tense.

We split the original Step 1.2. into two steps to keep the 4-line limit and change the text to use the imperative tense.

5. 1.3: Please ensure that all text is written in the imperative tense.

Changed, it's now Step 1.4 and 1.5.

6. 1.5: Please ensure that all text is written in the imperative tense.

Changed, it's now Steps 1.8.

7. 2.1: What's the cell density?

The required cell confluency added to the step 2.2.

8. 3.8: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

We combined the section 3.8 Tips and troubleshooting with the original section 5.5. Tips and troubleshooting and moved the merged text to the end of Discussion.

9. 3.8.1-3.8.4: Please ensure that all text is written in the imperative tense.

Done

10. 5.4: Please ensure that all text is written in the imperative tense.

Done