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FISH protocol for myotonic dystrophy type 1 cells

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Abstract

Mutant *DMPK* transcripts containing expanded CUG repeats (CUGexp) are retained within the nucleus of myotonic dystrophy type 1 (DM1) cells as discrete foci. Nuclear CUGexp-RNA foci that sequester MBNL1 splicing factor represent a hallmark of this RNA dominant disease caused by the expression of expanded microsatellite repeats. Here we described fluorescent *in situ* hybridization (FISH) techniques to detect either RNA containing CUG expansion or *DMPK* transcripts in human DM1 or WT cells. In addition, we propose a combined FISH/immunofluorescence protocol to visualize the colocalization of MBNL1 with CUGexp-RNA foci in DM1 cells.

Keys word: myotonic dystrophy, nuclear RNA foci, CUG expansion, *DMPK* transcripts, MBNL1, fluorescent *in situ* hybridization, immunofluorescence

1. Introduction

Myotonic dystrophy type 1 (DM1 or Steinert's disease) is a multisystemic disorder characterized by myotonia, progressive muscle weakness and atrophy, cardiac conduction defects and cognitive impairment together with other endocrine dysfunctions (1, 2). The disease is caused by an abnormal expansion of a CTG repeated sequence within the 3'untranslated region (UTR) of the Dystrophia Myotonica Protein Kinase (*DMPK*) gene (3-5). The size of the CTG tract is lower than 37 repeats in healthy population, but can reach several thousands in DM1 patients. Moreover, CTG expansions in patients are instable at both the somatic and intergenerational levels (6). Since the identification of this expanded microsatellite repeats, DM1 has been described as the archetype of RNA gain-of-function diseases. The mutant *DMPK* allele is transcribed, but messenger RNAs containing expanded CUG repeats (*CUGexp-DMPK*) are retained into the nucleus and form discrete aggregates or foci (7, 8). These ribonuclear foci sequester factors of the Muscleblind-like (MBNL) RNA binding protein family (9-11), resulting in their functional loss and thereby the disruption of RNA processing events including the misregulation of the alternative splicing of many effector genes (12-15). More than forty misregulated splicing events have been described in the skeletal muscle of DM1 patients and some of them were associated with symptoms (12, 16-20).

The finding of ribonuclear aggregates or RNA foci in DM1 cells was a major breakthrough in the understanding of the molecular disease process (7) and their presence in affected tissues of DM1 patients is currently a hallmark of the disease (11, 21, 22). Nuclear CUGexp-RNA foci were also detected in both *in vivo* and *in vitro* models of the disease, and their presence, number or intensity is often used as a readout to assess efficacy of

- 0.2% BSA
- 100nM CAG₇ fluorescent probe
- 7. PBS-T: RNase-free PBS + 0.1% Tween
- 8. PBS-MgCl₂: RNase-free PBS + 5mM MgCl₂
- 9. Hoechst solution: 2mg/mL of Hoechst in PBS-T
- 10. Anti-fading mounting medium for immunofluorescence

2.3. smiFISH

1. Glass slides
2. Microscope forceps
3. Dark chamber with flat bottom, moisten (ex: a 150mm petri dish with paper moisten with 30% formamide and 1xSSC)(see note 1)
4. Anti-fading mounting medium for immunofluorescence
5. Probe set targeting *DMPK* transcript (see table 1 for sequences and paragraph 3.3.1 for preparation)
6. Fluorescent FLAP probe: DNA oligonucleotide (see table 1 for sequence) conjugated to Cy3 fluorochrome at both extremities
7. smiFISH probe : solution of *DMPK* probe-set hybridized to fluorescent FLAP probe (see paragraph 3.3.2 for preparation)
8. FLAP hybridization reaction mix:
 - 4 μ M Probe-set
 - 5 μ M FLAP
 - 1x NEB3
 - H₂O
9. Pre-hybridization buffer:
 - 15% formamide,
 - 1x SSC
10. Hybridization reaction mix:
 - Mix 1:
 - 2x SSC
 - 0,68 μ g/ μ L *E.coli* tRNA
 - 30% formamide
 - 1:25 smiFISH probe (vol:vol)
 - H₂O
 - Mix 2:
 - 0,4mg/mL RNase free BSA
 - 4mM VRC (*Vanadylribonucleoside complex*)
 - 0,8 % Dextran sulfate
 - H₂O
11. RNase-free PBS
12. Hoechst solution: 2mg/mL of Hoechst in PBS-T

2.4. Immunofluorescence

1. Glass slides
2. Microscope forceps
3. Dark wet chamber
4. Monoclonal mouse primary antibody directed against MBNL1: MB1a (DSHB) **(31)**
5. Secondary antibody directed against mouse IgG (H+L) conjugated to a fluorochrome
6. Anti-fading mounting medium for immunofluorescence (ex: prolong gold)
7. BSA (powder)
8. PBS-T: PBS + 0.1% Tween

9. CSK buffer:
 - 100 mM NaCl
 - 300 mM Sucrose
 - 10mM PIPES, pH 6.8
 - 3 mM MgCl₂
10. Blocking buffer: PBS, tween 0.1%, 3% BSA
11. Hoechst solution: 2mg/mL of Hoechst in PBS-T

3. Methods

3.1 Preparation of cells for hybridization and immunofluorescence

DM1 cells (myoblasts or fibroblasts) are grown on glass coverslips coated with gelatin:

3.1.1. Preparation of coverslips and coating

1. Place a glass coverslip in the bottom of a multi-well plate or a petri dish using forceps.
2. Add a gelatin solution to cover the coverslip (ex. 1ml/35mm petri) and incubated for 1h at RT.
3. Rinse twice with PBS.
4. Plate the cells at the required density (see Note 2).

3.1.2. Cell growth and fixation

1. Cells are grown at 37°C and 5%CO₂.
2. Let the cells grow to the desired confluence (80% for proliferation or 100% for differentiation) (see Note 3).
3. Remove medium (see Note 4).
4. Carefully rinse the cells once with PBS.
5. Fix the cells with a solution of 4% PFA under chemical hood for 10 min (see Note 1).
6. Carefully rinse the cells twice with PBS.
7. Rinse the cells with EtOH 70%.
8. Incubate the cells overnight in EtOH 70% at 4°C (For convenience, cells/coverslips can be transferred to another multi-well plate using forceps).

3.2. Fluorescent *in situ* hybridization

3.2.1. Before starting:

1. See Note 5 for the standard incubation temperature.
2. Do not forget to work with RNase-free solutions.
3. Turn on a hybridization oven at 37°C and another at 45°C.
4. Warm 50 mL of PBS-T at 45°C.
5. Prepare the wet chamber for incubation/hybridization.

3.2.2. Hybridization:

1. Carefully wash twice the cells with PBS-MgCl₂ for 10 min.
2. Under the chemical hood: Prepare the hybridization buffer (ex. consider 20µl per 12mm coverslip, 50µL for 30mm)(see Note 1).
3. Place a piece of parafilm on the bottom of the chamber.

4. Dispose a drop of hybridization buffer on the parafilm .
5. Place the coverslip over the drop with the cells down.
6. Seal the incubation chamber with parafilm to avoid formamide fumes.
7. Incubate the cells in the chamber at 37°C for 90 min in a hybridization oven, in the dark (see note 6).
8. Under the chemical hood, replace the cells/coverslip in a multi-well plate using forceps.
9. Wash the cells once with PBS-T for 5 min at RT.
10. Wash the cells one with warmed PBS-T in a hybridization oven at 45°C for 30 min.
11. Incubate the cells once with Hoechst solution to counterstain the nucleus for 10 min.
12. Wash the cells twice with PBS-T for 10 min.
13. Mount the cells with an anti-fading mounting media on slide and stock the cells at least one night before visualizing the cells at the microscope (**Figure 1**).

3.3. smiFISH

Single Molecule inexpensive FISH or smiFISH is a technique allowing the visualization and quantification of single mRNA molecules using gene-specific unlabelled probe set hybridized (*in vitro*) to an fluorochrome-labelled secondary detector oligonucleotide (FLAP)(**Figure 2A**).

3.3.1. Design and preparation of DMPK probe set

1. The probes used to target *DMPK* transcripts using smiFISH protocol were designed using Oligostan script included in Fish_Quant package available at https://bitbucket.org/muellerflorian/fish_quant (**29**). The probes consist of 26 to 30 nucleotide gene-specific sequences coupled to a TTACTCTGGACCTCGTCGACATGCATT sequence, complementary to the FLAP secondary oligonucleotide (**Table 1**).
2. The 24 probes target the coding region of *DMPK* transcripts (**Figure 2B**).
3. The 24 oligonucleotide probes are resuspended TE buffer at a stock concentration of 100µM.
4. Prepare an equimolar mixture of the 24 oligonucleotide probes (concentration of each individual probe 4.16µM).
5. Dilute the probe mixture 5 times in TE buffer to get a 20µM probe solution (final concentration of each individual probe 0.83µM).
6. The probe-set is kept at -20°C for long-term storage.

3.3.2. Preparation of smiFISH probe

1. Prepare the FLAP hybridization reaction mix (ex. consider 20µl per 12mm coverslip, 50µL for 30mm) in the dark (see Note 6).
2. Incubate the reaction mix in a heating block at 85°C for 3 min.
3. Turn off the heating block and allow the tubes to cool down within the block until 35°C.
4. Put the samples on ice.
5. The hybridized duplexes (smiFISH probes) must be kept at -20°C in aliquots for long-term storage.

3.3.3. in situ hybridization

1. Prepare the cells (culture, fixation PFA 4% and permeabilization EtOH 70%) as

- previously described in section 3.1.2.
2. Wash the cells twice in PBS for 10 min.
 3. Under chemical hood: Incubate the cells at RT for 15min in pre-hybridization buffer.
 4. During the incubation prepare hybridization buffer by mixing equal volume of Mix 1 and Mix 2.
 5. Place a piece of parafilm on the bottom of the wet chamber.
 6. Dispose a drop of hybridization buffer on the parafilm.
 7. Place the coverslip over the drop with the cells down.
 8. Seal the incubation chamber with parafilm to avoid formamide fumes.
 9. Incubate the cells in the chamber at 37°C overnight in a hybridization oven in the dark (see Note 6).
 10. Under the chemical hood, replace the cells/coverslip in a multi-well plate using forceps.
 11. Wash the cells twice with freshly prepared pre-hybridization buffer for 30 min.
 12. Incubate the cells once with Hoechst solution for 10 min.
 13. Wash the cells twice with PBS-T for 10 min.
 14. Mount the cells with an anti-fading mounting media on slide and stock the cells at least one night before visualizing the cells at the microscope (**Figure 2C**).

3.4. MBNL1 immunofluorescence

3.4.1. Fixation:

Cells are prepared and fixed as previously described in 3.1.2. with a 4% PFA solution. In alternative method cells are fixed (and permeabilized) with an -20°C pre-chilled acetone: methanol mix for 10 min at RT and washed twice with PBS-T for 10 min (as described in Holt *et al.* 2009 **(31)**).

3.4.2. Permeabilization :

Several permeabilization methods can be used to visualize MBNL1 in human myoblasts and fibroblast depending on the state of differentiation of cells.

1. For undifferentiated/mono-nucleated cells:
 1. Proceed with PFA fixation as previously described.
 2. Rinse the cells with 70% ethanol solution.
 3. Incubate the cells overnight in 70% ethanol solution at 4°C.
2. For differentiated cells/myotubes:
 - a) CSK buffer before fixation
 1. Carefully discard the differentiation medium.
 2. Rinse the cells with 4°C chilled PBS on ice.
 3. Incubate the cells with CSK buffer for 3 min at 4°C.
 4. Rinse the cells once with PBS.
 5. Proceed with PFA fixation as previously described.
 - b) *Alternative:* Triton 1% after fixation
 1. Proceed with PFA fixation as previously described.
 2. Incubate the cells with a solution of PBS containing 1% triton for 15 min.

3.4.3. Blocking :

1. Rinse the cells twice with PBS-T.
2. Incubate with blocking buffer for 1h.
3. Prepare a wet chamber with a piece of parafilm in the bottom of the chamber.

3.4.4. Primary antibody:

1. Dilute the primary anti-MBNL1 antibody in blocking buffer (1/200e **(31)**).
2. Dispose a drop of diluted antibody on the parafilm (as example, 10-20 μ l of antibody per 12mm diameter coverslip, 50 μ l for a 30mm).
3. Place the coverslip over the drop with the cells down.
4. Incubate with primary antibody in a wet chamber for 1h.
5. Replace the cells/coverslip in the multi-well plate.
6. Wash the cells three times with PBS-T for 10 min.

3.4.5. Secondary antibody and mounting

1. Dilute the fluorochrome-conjugated secondary antibody in blocking buffer (1/400 - 1/500 with standard commercially available secondary antibodies).
2. Dispose a drop of diluted antibody on the parafilm (as example, 10-20 μ l of antibody per 12mm diameter coverslip, 50 μ l for a 30mm).
3. Place the coverslip over the drop by putting the cells down.
4. Incubate the cells with secondary antibody in the dark for 1h (see Note 6).
5. Replace the cells/coverslip in the multi-well plate.
6. Incubate the cells 10 min with a Hoechst solution.
7. Wash the cells twice with PBS-T for 10min.
8. Mount the cells with an anti-fading mounting media on slide and stock the cells at least one night before visualizing the cells at the microscope (**Figure 3A**).

3.5. Combined FISH and immunofluorescence experiments

For combined FISH/smiFISH-IF experiments (**Figure 3B**), the immunofluorescence protocol starts just before the incubation with the Hoechst solution (in both the FISH and the smiFISH protocols) and is done as described in section 3.4.3 and subsequent steps (see note 7).

4. Notes

1. Every manipulation involving formaldehyde-, formamide- or methanol-containing buffer should be performed under a chemical hood. This includes fixation of cells, preparation of hybridization buffers and hybridization steps.
2. For a homogenous repartition of the cells upon the surface of the petri, plate at high density (ex. for immortalized myoblasts: 30000 cells/cm²) and let them grow for 24-48hours before fixation or switch for differentiation.
3. To induce differentiation: When the cells reached 100% confluence, remove the GM, rinse the cells once with DMEM and replace with DM. When myotubes are formed (usually between 3 and 5 days post-differentiation) fix the cells.
4. The cells do not attach well on glass, so washing or medium changes must be done

carefully to prevent cell detachment, especially for myotubes. Other coating substrates like collagen-I or Matrigel may be used instead of gelatin.

5. All incubations or washing steps should be performed at room temperature (RT) unless otherwise indicated.
6. Incubation involving fluorochrome (oligonucleotide probes or antibodies) should be performed in the dark to prevent fading of the fluorochrome.
7. The permeabilization should be adapted in accordance to the protocol used. In the case of Triton 1% permeabilization, this step could be done after the FISH/smiFISH staining.

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Figure Legends :

Figure 1: RNA aggregates (or foci) of *CUGexp-DMPK* transcripts were detected by FISH using a Cy3-(CAG)⁷ (red) in the nucleus (Hoechst, blue) of DM1 muscle cells .

Figure 2: single molecule inexpensive FISH. A) schematic representation of the smiFISH principle. An ensemble of 24 gene specific probe are hybridized in vitro to a fluorescent secondary probe via the FLAP sequence. The resulting smiFISH probes are subsequently hybridized to the targeted transcripts in cells (adapted form Tsanov *et al.* 2016). **B)** Schematic representation of the 24 oligonucleotides probes on the *DMPK* transcripts. **C)** Representative image of *DMPK* transcripts (red) observed using smiFISH technique in differentiated human WT muscle cells (nucleus in blue).

Figure 3: Localisation of MBNL1 in WT and DM1 myoblasts. A) Visualization of MBNL1 (in green) using immunofluorescence technique in differentiated human WT muscle cells (nucleus in blue). **B)** Visualization of both mutated *DMPK* transcripts using FISH technique (CAG7 probe, red) and MBNL1 using immunofluorescence technique (MB1a antibody, green) in the nucleus of DM1 myoblast (Hoechst , blue)

Table 1 : sequence and modification of the probes used in FISH and smiFISH protocols

Protocol	Name	Sequence 5' -> 3'
<i>FISH</i>	CAG sequence	Cy3-CAGCAGCAGCAGCAGCAGCAG
<i>smiFISH</i>	DMPK-Probe 1	TCCGGGGGACACAGCAACCGCTGAATTTACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 2	ACAGGTAGTTCTCATCCTGGAAGGCGAAGTTTACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 3	CAGTCGGACCTCCTTAAGCCTCACCATTACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 4	CAACTGCCGGACGTGTGCCTTAGGTTTACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 5	TTCTGGTTGTCCGTGCGGATGGCCTTACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 6	CGCTGGAAGTCCACTTCAGCTGTTTTTACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 7	CTGTCCCTGAGGGCCATGCAGGAGTATTACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 8	GTGAGCCCGTCTCCACCAAGTGAATTACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 9	TGCATGTGTCGGTGGACCTTCGAAATCTTACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 10	GCTCCTGTAGTGGACGATCTTGCCATTTACTCGGACCTCGTCGACATGCATT

	DMPK-Probe 11	GCGTCTGCCCATAGAACATTTTCATAGGCTTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 12	CATCTGCCCCGAGCTTGAGGCAAGAGTTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 13	GCCACAGCGGTCCAGCAGGATGTTGTTTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 14	GGTTTGATGTCCCTGTGCACGTAGCCATTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 15	CCGCCCACGTAATACTCCATGACCAGTTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 16	CGTCTGCTTCATCTTCACTACCGCTATTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 17	GCCCACTGCAAGAAGTCGGCCACGTATTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 18	CGCACCTTCCC GAATGTCCGACAGTGTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 19	GTCTCCGCCGTGGAATCCGCGTAGAATTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 20	CTCCCCAACTTGCTCAGCAGTGTTCATTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 21	CCCTCTTCAGCATGTCCCACTTGTTCTTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 22	CCCGCGTCCGATCACCTTCAGAATCTTTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 23	ACCATCCCAGTCGAGGCCAAAGAAGGTTACACTCGGACCTCGTCGACATGCATT
	DMPK-Probe 24	AGTAGCCACAAAAGGCAGGTGGACCCTTACACTCGGACCTCGTCGACATGCATT
<i>smiFISH</i>	FLAP sequence	Cy3-AATGCATGTCGACGAGGTCCGAGTGTA-Cy3

Figure 1

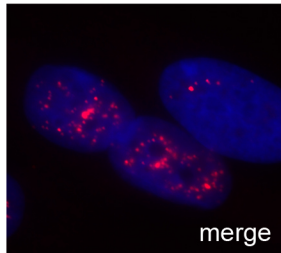
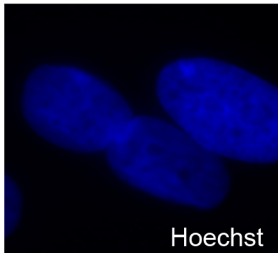
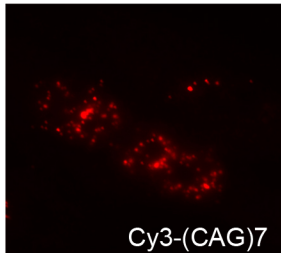
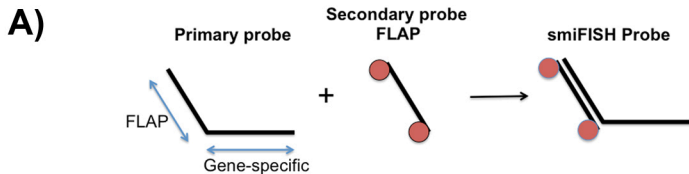


Figure 2



DMPK mRNA + 24 primary probes :

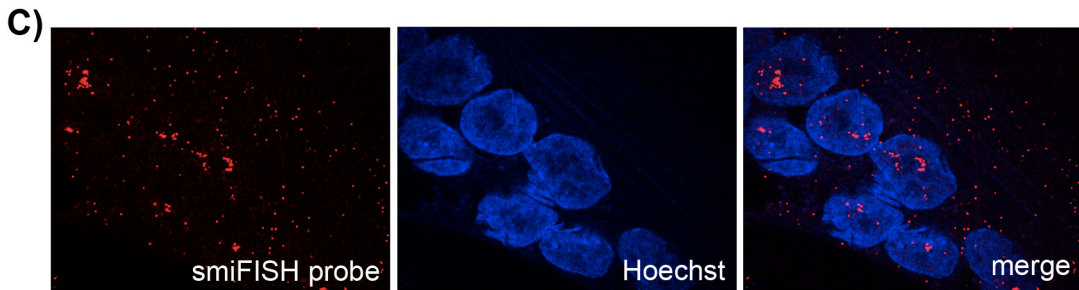
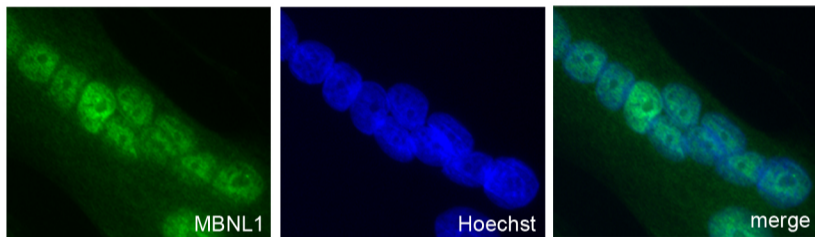


Figure 3

A)



B)

