# Chapter 2

# In Vivo Magnetic Resonance Imaging of Small Interfering RNA Nanodelivery to Pancreatic Islets

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## Abstract

Pancreatic islet transplantation is a promising therapeutic approach for type 1 diabetes.

However, recent advances in islet transplantation are limited by significant graft loss after transplantation. Multiple immunological and nonimmunological factors contribute to this loss. Novel therapies that could target the core reasons for the islet graft loss are desperately needed. Small interfering RNA can be used to inhibit the expression of virtually any gene with single-nucleotide specificity including genes responsible for islet damage. Applying adequate delivery of siRNA molecules to pancreatic islets prior to transplantation holds a great potential for improving the survival of islet grafts. Noninvasive imaging provides means for monitoring the survival of transplanted islets in real time. Here, we summarize the approach that has been developed to deliver siRNA to pancreatic islets in conjunction with tracking of the graft outcome by in vivo magnetic resonance imaging (MRI). We synthesize a nano-sized theranostic agent consisting of magnetic nanoparticles (MN), a reporter for MRI, labeled with Cy5.5 dye for near-infrared fluorescence (NIRF) imaging, and conjugated to siRNA molecule targeting genes that are harmful to islet grafts. Pre-labeling of islets by MN-Cy5.5-siRNA allowed us to monitor the survival of transplanted islet grafts by MRI and NIRF imaging and resulted in efficient silencing of the target genes in vivo. This novel approach combines a therapeutic effect provided by RNA interference technology with in vivo MR imaging and is expected to significantly improve the outcome of islet transplantation in patients with type 1 diabetes.

**Key words** Pancreatic islet, Small interfering RNA, Molecular imaging, Magnetic nanoparticles, Diabetes

## 1 Introduction

Type 1 diabetes (T1D) is a severe disease that results from destruction of insulin-producing pancreatic beta cells by autoimmune attack [1]. It is established that insulin injections do not cure T1D, although they could change the clinical course of T1D from an acutely fatal disease to a chronic one with long-term complications [2]. Currently, there are two strategies for the etiological treatment of diabetes: immunoregulatory therapy and pancreas or islet transplantation [3, 4]. The toxic effect of most anti-diabetic drugs,

Zdravka Medarova (ed.), *RNA Imaging: Methods and Protocols*, Methods in Molecular Biology, vol. 1372,
DOI 10.1007/978-1-4939-3148-4_2, 

combined with the risk associated with immune suppression, limits their use. Elevated risk of surgical complications and relative invasiveness of the procedure make the practice of solid organ transplantation rare in T1D patients. Islet transplantation has emerged as one of the most promising therapeutic approaches for T1D treatment during the recent decade, but its success is hampered by drastic graft loss during the first several weeks after transplantation, due to multiple immunological and nonimmunological factors [5]. Therefore, there is an urgent need for developing strategies and methods that could protect islet grafts from abovementioned damages.

Small interfering RNAs, which are molecules that mediate an innate cellular mechanism for posttranscriptional regulation of gene expression, have a great potential for islet graft protection by silencing the expression of harmful genes with single-nucleotide specificity [6]. Magnetic resonance imaging provides the necessary set of tools for in vivo monitoring of transplanted islets and assessment of protection provided by siRNA. Recent studies from our group have demonstrated the coupling of siRNA to MRI contrast agents that can be used for noninvasive imaging [7–10].

Here, we summarize the methodology of a magnetic iron oxide nanoparticle platform for the delivery of siRNA to pancreatic islets followed by monitoring of the graft outcome by MRI and near-infrared optical imaging. This theranostic complex consists of magnetic nanoparticles that serve as a reporter for MR imaging, labeled with Cy5.5 dye for NIRF imaging, and conjugated to siRNA molecule targeting model or therapeutic genes of interest. The described approach includes three major steps: (1) synthesis of the MN-Cy5.5-siRNA complex, (2) pre-labeling islets with MN-Cy5.5-siRNA complex in vitro followed by islet transplantation under the kidney capsule of diabetic mice, and (3) real-time noninvasive MRI and NIRF imaging of transplanted islet grafts and assessing their survival.

## 2 Materials

### 2.1 Synthesis of MN-NIRF-siRNA Complex

The syntheses of MN-Cy5.5 and MN-Cy5.5-siRNA have been described previously [7–10].

1. Amino-derivatized dextran-coated iron oxide magnetic nanoparticles (pH 8.5).
2. 0.5 M Sodium bicarbonate, pH 9.6.
3. Cy5.5 NHS ester, 1 mg (Amersham Biosciences, Piscataway, NJ).
4. 20 mM Sodium citrate, 150 mM sodium chloride, pH 7, 7.5, 8.
5. Sephadex G-25, PD-10 column (Amersham Biosciences, Piscataway, NJ).

6. *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pierce Biotechnology, Rockford, IL).
7. 50 mM Sodium phosphate, 10 mM EDTA, pH 7.5, 8.
8. 15 mM and 35 mM Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP, Thermo Fisher Scientific, Rockford, IL).
9. Double-stranded siRNA modified with a thiol group on the 5′ end of the sense strand (Dharmacon, Lafayette, CO).
10. Quick Spin Column G-50 Sephadex column (Roche Applied Science, Indianapolis, IN).
11. The 20 μ magnetic separation columns and MACS separator (Miltenyi Biotec, Inc., Auburn, CA) (catalog number: 130-042-701).
12. Agarose gel in Tris-borate-EDTA (TBE) buffer (Invitrogen, Carlsbad, CA).
13. Ethidium bromide (Sigma-Aldrich, St. Louis, MO).
14. Bruker MQ20 Minispec NMR spectrometer (Bruker Biospin Co., Billerica, MA).
15. Sub-micron particle size analyzer (Coulter N-4, Hialeah, FL).
16. SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA).
17. Bench-top centrifuge (Thermo Scientific).
18. Molecular Imager FX scanner (Bio-Rad Laboratories, Hercules, CA).

### 2.2 Pre-labeling Islets In Vitro Followed by Islet Transplantation

Labeling of pancreatic islets using MN-Cy5.5-siRNA probes and islet transplantation have been described previously [11, 7, 9, 10].

1. Pancreatic islets from healthy human donors (Integrated Islet Distribution Program, IIDP Centers, National Institutes of Health and Juvenile Diabetes Research Foundation).
2. CMRL 1066 medium, fetal bovine serum (FBS), and penicillin-streptomycin (GIBCO, Grand Island, NY).
3. 24-Well nontreated plates (Nunc, Roskilde, Denmark).
4. $CO_2$ incubator (Thermo Scientific), Class II type A2 biological safety cabinet (Thermo Scientific).
5. 37 % formaldehyde (Fisher Scientific, Rockford, IL).
6. Tris-EDTA buffer, pH 8.0 (Fisher Scientific, Rockford, IL).
7. 1× Phosphate-buffered saline (PBS) (Sigma-Aldrich, Milwaukee, WI).
8. Normal goat serum (Vector Laboratories, Inc. Barlingame, CA).

9. Guinea pig anti-human insulin primary antibody (Abcam, Cambridge, MA) and FITC-labeled goat anti-guinea pig secondary IgG (Abcam, Cambridge, MA).
10. DAPI (Vectashield, Vector Laboratories, Inc. Barlingame, CA).
11. 5 % potassium ferrocyanide (ACROS Organics, Fairlawn, NJ)
12. 5 % hydrochloric acid (Sigma-Aldrich, Milwaukee, WI).
13. Nuclear Fast Red (Sigma-Aldrich, Milwaukee, WI)
14. Mounting medium (Permount, Fisher Scientific, Fair Lawn, NJ).
15. Ethanol and xylene (Fisher Scientific, Rockford, IL).
16. Nikon Eclipse 50i microscope, SPOT 7.4 Slider RTKE CCD camera, and iVision 4.015 version software (Diagnostic Instruments, Sterling Heights, MI).
17. 9.4-T horizontal bore imaging unit equipped with a home-built radiofrequency transmit-receive 3 × 4-cm elliptical surface coil and ParaVision 5.1 Software (Bruker, Billerica, MA).
18. Marevisi 3.5 software (Institute for Biodiagnostics, National Research Council, Canada).
19. CellTiter 96 non-radioactive cell proliferation assay kit (Promega, Madison, WI).
20. SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA).
21. Human insulin ELISA kit (Mercodia, Uppsala, Sweden).
22. 5-Week-old NOD/scid mice (The Jackson Laboratory, Bar Harbor, ME).
23. Streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO).
24. Animal anesthesia system Isotec 4 (Surgivet/Anesco, Waukesha, WI).
25. Acensia Contour Blood Glucose Monitoring System (Bayer Health Care, Tarrytown, NY).
26. Standard surgery kit (scissors, forceps) (Roboz Surgical Instrument Co., Inc., Gaithersburg, MD).
27. Tuberculin syringes, 1 cc (Becton Dickinson, Franklin Lakes, NJ).

### 2.3 MR and NIRFImaging of Transplanted Islet Grafts

In vivo NIFR optical imaging and MRI of islet grafts under the kidney capsule of diabetic mice has been described previously [12, 13, 11, 9, 10].

1. Alfalfa-free diet (TestDiet, Richmond, IN).
2. Animal anesthesia system Isotec 4 (Surgivet/Anesco, Waukesha, WI).

3. Whole-body animal imaging system (IVIS Spectrum), equipped with 10 narrow-band excitation filters (30 nm bandwidth) and 18 narrow-band emission filters (20 nm bandwidth) (Perkin Elmer, Hopkinton, MA).
4. Living Image 4.2 software (Perkin Elmer, Hopkinton, MA).
5. 9.4-T horizontal bore imaging unit equipped with a home-built radiofrequency transmit-receive 3 × 4-cm elliptical surface coil and ParaVision 5.1 Software (Bruker, Billerica, MA).
6. Marevisi 3.5 software (Institute for Biodiagnostics, National Research Council, Canada).

## 3 Methods

The synthesis of siRNA-conjugated nano-probes consists of two steps: conjugation of the fluorescent dye Cy5.5-NHS ester to dextran-coated MN followed by conjugation of siRNA to MN-Cy5.5. Labeling of human islets with MN-Cy5.5-siRNA and transplantation under the kidney capsule of diabetic animals is explained in Subheading 3.2. Monitoring of transplanted islets grafts by both MRI and NIRF imaging is depicted in Subheading 3.3.

### *3.1 Conjugation of Cy5.5 -NHS Ester to MN and Conjugation of siRNA to MN-Cy5.5*

1. The pH of amino-derivatized dextran-coated iron oxide MN is adjusted to 9.6 with 0.5 M sodium bicarbonate.
2. A solution of monoactivated Cy5.5-NHS ester (1 mg) in 100 μL DMSO is reacted with MN (~10 mg Fe) in 20 mM sodium citrate and 150 mM sodium chloride with constant agitation for 12 h at room temperature.
3. The Cy5.5-labeled aminated iron oxide (MN-Cy5.5) is purified from nonreacted dye using a Sephadex G-25, PD-10 column equilibrated with 20 mM sodium citrate, 150 mM sodium chloride, pH 7.5.
4. MN-Cy5.5 is conjugated to the heterobifunctional cross-linker SPDP via N-hydroxy succinimide ester. The intermediate is purified using a Sephadex G-25, PD-10 column in a buffer containing 50 mM sodium phosphate, 150 mM sodium chloride, 10 mM EDTA, pH 7.5.
5. Prior to labeling, the disulfide protecting group on 5′-*S*-*S*-(CH2)6 of the custom-synthesized siRNA duplex is deprotected using 35 mM TCEP. The free thiol-siRNA is then reacted with MN-Cy5.5 via the SPDP crosslinker in 50 mM sodium phosphate, 150 mM sodium chloride, 10 mM EDTA, pH 8.0 at 4 °C over night. The product is purified using a Quick Spin Column G-50 Sephadex column.
6. Free siRNAs is removed from MN-siRNA-Cy5.5 solution using a magnetic separation column. The 20 μ column is attached to a MACS separator and washed with 20 μl of 50

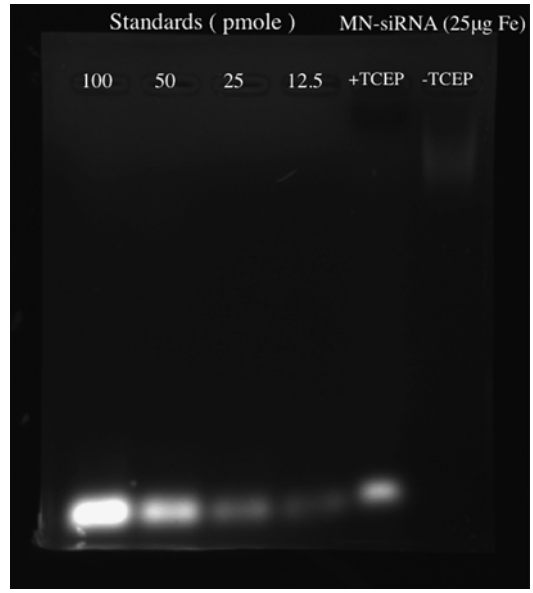


**Fig. 1** Agarose gel electrophoresis shows that the siRNA molecules conjugated to the MN-Cy5.5-siRNA probes were cleaved using treatment with a reducing agent TCEP

mM sodium phosphate, 150 mM sodium chloride, 10 mM EDTA, pH 8.0. The sample is applied onto the column, and the column is rinsed with 50 μl of 50 mM sodium phosphate, 150 mM sodium chloride, 10 mM EDTA, pH 8.0, to let the free siRNA pass through. Then the column is removed from the separator. Twenty microliters of 50 mM sodium phosphate, 150 mM sodium chloride, and 10 mM EDTA, pH 8.0 is applied onto the column and the siRNA conjugated probe is flushed out and collected.

7. The labeling ratio of Cy5.5 per MN crystal is obtained spectrophotometrically. The dye-to-particle ratio is calculated from the concentrations of Cy5.5 and iron. For the Cy5.5 dye, the number of dyes per particle is obtained using absorption at 678 nm ($e = 250 \times 10^3$ $M^{-1}$ $cm^{-1}$). Iron concentration is determined spectrophotometrically from absorption at 410 nm (*see* **Note 1**).
8. The number of SPDP molecules per crystal is determined based on the release of pyridine-2-thione at 343 nm ($e = 8.08 \times 10^3$ $M^{-1}$ $cm^{-1}$) after the addition of 35 mM of TCEP in PBS buffer. The amount of conjugated siRNA is assayed using agarose gel electrophoresis. The amount of siRNA dissociated from the nanoparticles is assessed under reducing conditions by pretreatment with 15 mM TCEP in RNase-free PBS buffer for 30 min. siRNA standards, untreated probes, and probes treated with a reducing agent are applied to a 2 %

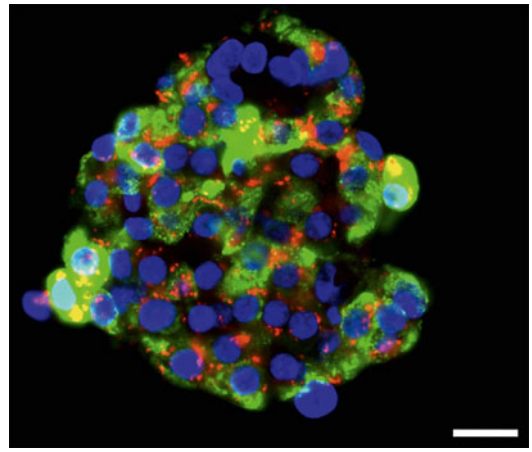

**Fig. 2** Islets were treated with MN-Cy5.5-siRNA for 48 h. Fluorescence microscopy of the labeled islets shows heavy labeling of islet cells with MN-Cy5.5-siRNA probes. *Green*: insulin stain; *red*: Cy5.5 conjugated to the nanoparticles; *blue*: cell nucleus (magnification bar = 15 μm)

agarose gel in RNase-free TBE buffer and run at 145 V for 1 h. The gel is stained with 0.5 mg/mL ethidium bromide for 30 min, and visualized using a Molecular Imager FX scanner (Fig. 1) (*see* **Note 2**).

### *3.2 Human Islet Labeling with MN-Cy5.5-siRNA and Transplantation under the Kidney Capsule*

1. Human islets are cultured in 24-well nontreated plates at 1000 islet equivalents (IEQ)/well in CMRL 1066 medium supplemented with 10 % FBS and 100 mg/mL penicillin-streptomycin (*see* **Note 3**). For labeling experiments, 1000 IEQ are incubated with MN-Cy5.5-siRNA containing cell culture medium (25 mg Fe/mL) for 48 h at 37 °C in a $CO_2$ incubator (*see* **Notes 4** and **5**).
2. Labeled pancreatic islets are fixed in 4 % formaldehyde for 10 min and spun down by centrifugation at 2000 rpm for 5 min. Then islet pellets are suspended in 100 μl of 1 % warm agarose/1× PBS solution at 60 °C. Next, the agarose-islet suspension is fixed with 4 % formaldehyde overnight, followed by paraffin embedding and tissue sectioning (*see* **Notes 6** and **7**).
3. Fluorescent immunostaining and Prussian Blue iron staining of the islets incubated with MN-Cy5.5-siRNA are used to identify the presence of nano-probes in the islets. The slides are deparaffinized and rehydrated. For fluorescent immunostaining (Fig. 2), after antigen retrieval with Tris-EDTA buffer (pH 8.0) in microwave oven at high for 10 min and blocking with 5 % goat serum in 1×PBS at room temperature for 1 h, the slides are incubated with guinea pig anti-human insulin primary antibody (1:50 dilution) at 4 °C overnight, followed by incubation with a FITC-labeled goat anti-guinea pig second-

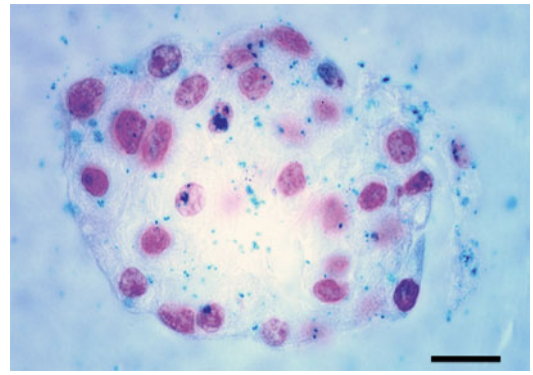

**Fig. 3** Islets were treated with MN-Cy5.5-siRNA for 48 h. Prussian Blue staining demonstrates that islet cells are labeled with the iron oxide nanoparticle probes. *Blue*: iron oxide nanoparticles; *red*: cell nucleus (magnification bar = 15 μm)

ary IgG (1:100 dilution). Next, the slides are washed three times, counterstained, and mounted with mounting medium with DAPI. For Prussian Blue iron staining (Fig. 3), the sections are washed in 1×PBS for 5 min, and incubated in Prussian Blue solution containing 5 % potassium ferrocyanide and 5 % hydrochloric acid at RT for 1 h. Next, the slides are rinsed in double-distilled $H_2O$ for 30 min followed by staining with Nuclear Fast Red for 5 min. Subsequently, the sections are dehydrated with graded ethanol and cleared with 100 % xylene. After drying overnight, the slides are mounted in 10 μl Permount mounting medium.

4. The sections stained for immunofluorescence and Prussian Blue iron stain are examined using Nikon Eclipse 50i microscope. Images are acquired using a CCD camera and analyzed with iVision 4.015 version software.
5. Islet phantoms for in vitro MRI are prepared by fixing islet pellets in 2 % formaldehyde and sedimenting them in 1 % agarose gel in Eppendorf tubes. Imaging is performed using a 9.4 T scanner equipped with ParaVision 5.1 software. The imaging protocol consisted of coronal T2-weighted spin echo (SE) pulse sequences with the following parameters: repetition time (TR)/echo time (TE) = 3000/8, 16, 24, 32, 40, 48, 56, 64 ms, field of view (FOV) = 3.2 $cm^2$, matrix size 128, resolution 250 $mm^2$, and slice thickness = 0.5 mm. T2 relaxation times are determined by T2 map analysis of regions of interest drawn around the islet pellets using Marevisi 3.5 software.
6. Diabetes in 5-week-old NOD/scid mice is induced by intraperitoneal injections of STZ (200 mg/kg body weight) freshly dissolved in sodium citrate buffer (*see* **Notes 8** and **9**). MN-Cy5.5-siRNA labeled human pancreatic islets are implanted

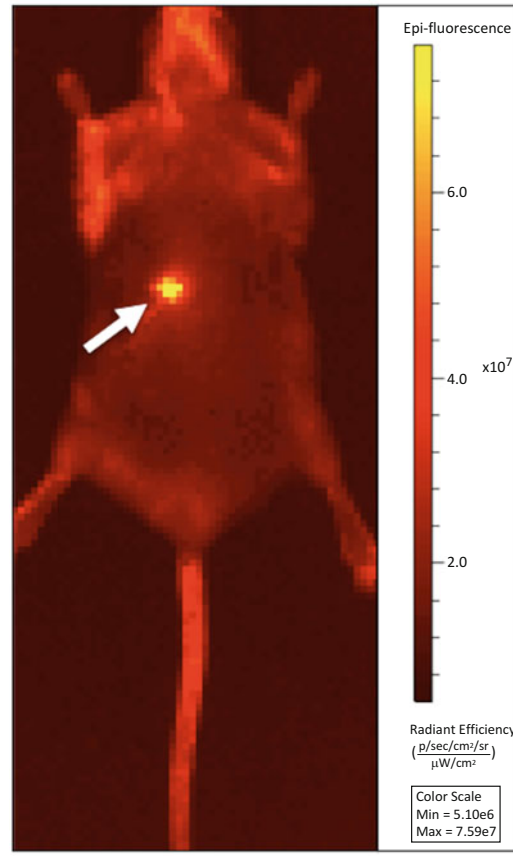


**Fig. 4** In vivo near infrared optical imaging of islet grafts (*arrow*) under the left kidney capsule 3 days post-islet transplantation. The bright spot indicates the presence of Cy5.5 signal from the islet grafts labeled with MN-Cy5.5-siRNA

under the left kidney capsule (1500 IEQ/kidney) of NOD/scid mice. Normoglycemia is restored in all animals 2–3 days after transplantation (*see* **Note 10**).

### *3.3 In Vivo Optical and MR Imaging of Islet Grafts*

1. Blood glucose levels of recipient mice are monitored using an Acensia Contour Blood Glucose Monitoring System twice weekly starting before the STZ injections and extending 7 weeks after islet transplantation.
2. In vivo optical imaging of islet grafts and 3D reconstruction

   All animals are fed with an alfalfa-free diet 2 weeks before islet transplantation (*see* **Note 11**). In vivo optical imaging is performed 7, 14, 21, 28, 35, 42, and 49 days after islet transplantation using an IVIS Spectrum whole-body animal imaging system. The Cy5.5 signal from the recipient mice is collected at 640 nm excitation and 720 nm emission (Fig. 4). The origin of

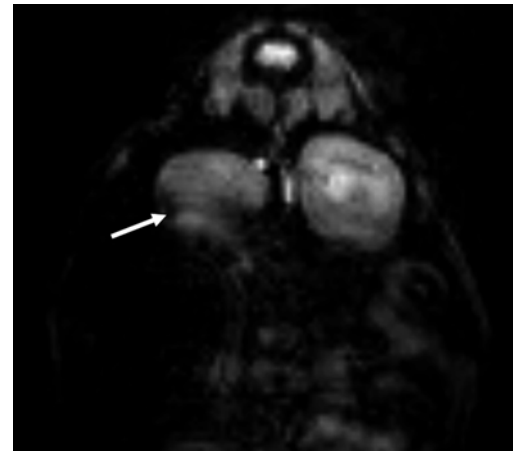

**Fig. 5** T2-weightedMRI of the graft labeled with MN-Cy5.5-siRNA. MRI detects the islet grafts as a pocket-shaped signal void under the left kidney capsule (*arrow*)

the Cy5.5 signal in the animals is validated by 3D reconstruction using Living Image 4.2 software.

3. In vivoMRI of transplanted human islet grafts

   In vivo MRI of the recipient mice is performed 7, 14, 21, 28, 35, 42, and 49 days after islet transplantation using a 9.4-T scanner equipped with ParaVision 5.1 software. The imaging protocols consist of multi-slice multiecho T2-weighted map (for volume and T2 relaxivity measurement). Parameters: TR = 2000 ms and multiecho TE = 8, 16, 24, 32, 40, 48, 56, and 64; number of averages (NA) = 4; rapid acquisition with relaxation enhancement factor = 8; FOV = 4.4 $cm^2$, matrix size 128, spatial resolution 312 $mm^2$, and slice thickness = 0.5 mm. T2-weighted images are analyzed on a voxel-by-voxel basis by fitting the T2 measurements to a standard exponential decay curve, defined by the equation: [y = Aexp (-t/T2)]. Graft volumes under the kidney capsule are calculated by counting the number of voxels in each slice of a region of interest (ROI) outlining the graft and multiplying by the voxel volume (0.05 $mm^3$) using Marevisi 3.5 software (Fig. 5) (*see* **Note 12**).

## 4 Notes

1. The relaxivity values of the probes are determined at 37 °C using a Mini spec

   NMR spectrometer and the measurements of the longitudinal (R1) and transverse (R2) magnetization properties of the sample are represented as $mmol^{-1}$ $s^{-1}$ for a given temperature.

The size and zeta potential of MN-Cy5.5-siRNA are measured using dynamic light scattering.

2. The selection of optimal magnetic separation column depends on the sample size and applications. The entire synthesis including the agarose gel electrophoresis has to be completed under RNase-free conditions.
3. Both the viability and purity of the islets used for the experiments should be higher than 85 %.
4. The viability of islets incubated with MN-Cy5.5-siRNA is determined by colorimetric (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using CellTiter 96 non-radioactive cell proliferation assay kit. After the treatment with MN-Cy5.5-siRNA probes for 48 h, islets are washed with culture medium two times, and resuspended in a 24 well non-treated plate followed by addition of 150 μl of a tetrazolium dye solution. After incubation at 37 °C for 4 h, 1 ml of solubilization solution/stop mix is added to each well to dissolve the dark blue crystals. After overnight incubation, the solution is transferred to cuvette and read on SpectraMax M2 spectrophotometer ($\lambda_{test} = 570$ nm and $\lambda_{reference} = 630$ nm).
5. Insulin secretion is evaluated by glucose-stimulated insulin secretion assay using static incubation of MN-Cy5.5-siRNA labeled islets at low (1.7 mmol/L) and high (20 mmol/L) glucose concentrations. Insulin concentrations in supernatants are measured using a human insulin ELISA kit. A stimulation index is calculated as the ratio of stimulated to basal insulin secretion normalized by the insulin content.
6. We find that frozen sections of islet agarose pellets are more easily detached from the glass slides.
7. Silencing efficacy of the target genes in islets incubated with the probe can be determined by real-time RT-PCR (at mRNA level) and Western blot (at protein level).
8. The STZ solution needs to be freshly prepared every time immediately before the injection by dissolving STZ in a sodium citrate buffer.
9. Diabetes in experimental animals is confirmed by weight loss, polyuria, and blood glucose levels higher than 250 mg/dL as assessed by an Acensia Contour Blood Glucose Monitoring System.
10. Intrahepatic islet transplantation via portal vein is an alternative transplantation site that has been found to be effective. Each of these locations has their own advantages and disadvantages. Therefore, the selection of an islet transplantation site should be determined by the objectives of the study.

11. An alfalfa-free diet can decrease the autofluorescence signal originated from the intestinal area of the animal.
12. Islet grafts can be easily identified on the surface of the recipient's kidney. To demonstrate ex vivo target gene downregulation in the islets grafts, immunostaining of tissue sections can be used. In addition, the graft tissue can be dissected from the kidney and collected for ex vivo western blot analysis and RT-PCR.

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