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Analysis of the Intracellular Role of Galectins in Cell Growth and Apoptosis

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Abstract

Galectins are a family of animal lectins with conserved carbohydrate-recognition domains that recognize β-galactosides. Despite structural similarities, these proteins have diverse functions in a variety of cellular processes. While a large number of extracellular functions have been demonstrated for galectins, the existence of intracellular functions has been clearly shown for a number of galectins, including regulation of cell growth and apoptosis; these latter functions may not involve glycan binding. There is considerable interest in intracellular regulation by galectins of cell growth and apoptosis, as these are fundamental cellular processes in normal homeostasis. Their dysregulation can cause pathologies such as autoimmune disorders, cancer, and neural degenerative diseases. Here we describe methods that we routinely perform in the laboratory to investigate the role of galectins in cell growth and apoptosis. These include methods for cell isolation, cell maintenance, and genetic manipulations to perturb galectin gene expression, as well as assays for cell growth and apoptosis.

Keywords

Galectin; Intracellular regulation; Cell growth; Apoptosis

1 Introduction

The galectin family has presented an intriguing set of proteins located inside and outside the cell compartment. Early studies of galectin-1 and galectin-3 biology revealed predominant nuclear and cytoplasmic localization [9, 10], but externalization of galectins was also observed and was recognized to occur through unconventional secretion [9, 10]. Analyses of glycan recognition by galectin-1, -2, and -3 revealed ligand specificity that suggested differential extracellular functions could be attributed to each galectin [2, 3]. Almost a decade later, subsequent studies in a seminal report that described how recombinant galectin-1 could regulate T cell function through its ability to induce apoptosis in a carbohydrate-dependent manner by recognition of cell surface CD45 on activated T cells [4]. Along with its effect in causing cell death, galectin-1 was also observed to mediate adhesion to ECM components [5] and regulate cell growth [6, 7].

In the meantime, examination of the function of galectin-3 in T cells revealed that it possessed an intracellular function. When expressed, this lectin conferred resistance to apoptotic stimuli and beneficial influence on cell growth under suboptimal conditions [8].

Furthermore, galectin-3 was observed to interact directly with Bcl-2 in a manner that was sensitive to inhibition by lactose. As Bcl-2 is not known to be glycosylated these observations suggested that galectin-3 could interact with other proteins in a lectin-independent fashion. Although galectin-3 possesses a non-lectin N-terminal domain, this lectin appears to have the ability to function through peptide-peptide interaction with its lectin domain, and this mode of action may be a common property of galectins. Galectins are likely to perform intracellular functions because they are present in substantial quantities in intracellular compartments, including the nuclei and the subcellular localization (i.e., cytosol vs. nucleus), and corresponds to the proliferation status of the cell [9, 10].

Further investigations by others have since revealed other intracellular signaling partners of galectin-3, exemplified by gemin-4, K-Ras, β-catenin, and Alix [11 – 15]. Additionally, galectin-3 participates in cross-signaling with p53-induced apoptosis whereby the levels of galectin-3, as an anti-apoptotic protein, are reduced in order to coordinate p53 function [16]. The anti-apoptotic function of galectin-3 has since been observed in many independent studies and a variety of cell types (reviewed in [17, 18]). Interestingly, in contrast to anti-apoptotic galectin-3, galectin-7 is highly induced by p53 [19], and was described to possess an intracellular pro-apoptotic function that operates by activation of JNK and cytochrome *c* release [20]. In this regard, a prominent theme to intracellular functions of galectins may be their participation in the regulation of cell growth and survival. On the other hand, a large number of functions unrelated to cell growth and apoptosis have been described for galectins (reviewed in [21 – 28]).

Like galectin-1, galectin-3 has been observed to induce apoptosis in T cells, through cell surface glycan binding [29, 30]. Thus, galectin-3 inhibits apoptosis through an intracellular mechanism independent of glycan binding, while it promotes apoptosis through an extracellular glycan-binding mechanism. It is to be noted that other galectins, including galectin-2, -4, -8, and -9, also kill T cells, as well as other cell types [31 – 34], each likely acting by binding to distinct cell surface glycoproteins in accordance to their respective glycan specificities. Whether they also regulate apoptosis through intracellular mechanisms is unknown.

The protocols below describe the analyses of anti-apoptotic properties of galectin-3 in different cell types. Unactivated T cells express insignificant levels of galectin-3, and this is represented by the Jurkat E6-1 cell line. In this cell line, the function of galectin-3 was analyzed after transfection with a galectin-3 construct to induce ectopic expression. Studies in two other cell types are described—macrophages and keratinocytes. These cells are derived from galectin-3-deficient (KO) mice, which are available from the authors' laboratory.

2 Materials

2.1 Analysis of the Intracellular Role of Galectins in Cell Growth and Apoptosis by Altering Gene Expression Levels in Cell Lines

2.1.1 Examination of the Role of Galectin-3 Expression in Jurkat E6-1 Cells

1. Jurkat E6.1 cells (ATCC).

2. Human galectin-3 cDNA cloned into an eukaryotic vector conferring G418 resistance.
3. 0.25 M NaCl.
4. Sterile TE: 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.
5. 100 % ethanol.
6. 5 M NaCl stock.
7. 1 M Tris–HCl, 100 mM EDTA, pH 7.5 (100×).
8. Complete culture media: RPMI 1640 medium, 10 % FBS.
9. Incubator at 37 °C and 5 % CO_2.
10. Clinical centrifuge.
11. BioRad Gene Pulser electroporator.
12. G418 100 mg/ml in PBS.
13. Triton X-100.
14. Lysis buffer: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 140 mM NaCl, 1 % Triton X-100, 0.2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM 2-mercaptoethanol (commercial cocktail for eukaryotic cells, *see* Note 1).
15. Protease inhibitor stocks (20 mM PMSF/ethanol, 100 μg/ml leupeptin, 100 μg/ml pepstatin/DMSO).
16. Mercaptoethanol.
17. BCA assay (Pierce/Thermo Fisher).
18. Refrigerated microcentrifuge.
19. PVDF membrane (Immobilon P, Millipore).
20. PAGE apparatus and 12 % polyacrylamide gels.

2.1.2 Galectin-3 in HeLa Cells (ATCC)

1. siRNA (Stealth RNAi, Invitrogen) (*see* Notes 2 and [3]).
2. RNase-free water.
3. 1 M Tris–HCl, 100 mM EDTA, 2 M NaCl, pH 8.0 (100×).

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[1]Stock concentrations of inhibitors are 0.2 mM PMSF in absolute ethanol (due to limited stability, add to lysis buffer just before use and vortex thoroughly), 1 mg/ml pepstatin in dimethyl sulfoxide, and 1 mg/ml leupeptin in H_2O. The concentration of undiluted mercaptoethanol is 14 M. All reagents are available from Sigma.

[2]Order three pairs of complementary Stealth RNAi siRNA oligonucleotides from Invitrogen for control, and galectin-3. The three pairs of galectin-3 siRNA strands are as follows: CCAUGAUGCGUUAUCUGGGUCUGGA, UCCAGACCCAGAUAACGCAUCAUGG; UGCUGGGCCACUGAUUGUGCCUUAU, AUAAGGCACAAUCAGUGGCCCAGCA; UGGUGCCUCGCAUGCUGAUAACAAU, and AUUGUUAUCAGCAUGCGAGGCACCA. Dissolve in RNase-free water.

[3]The use of Stealth siRNA minimizes off-target effects. These siRNAs provide higher specificity and increased stability compared to standard siRNA.

4. HeLa cells (ATCC).
5. PolyFect or Effectene transfection reagent (Qiagen).
6. 24-Well culture plates.
7. Media: DMEM with 10 % FBS.

2.1.3 Assays of Cell Growth and Apoptosis in Cell Lines

1. MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] or CellTiter 96 Aqueous Cell Proliferation Assay kit (Promega).
2. Anti-human Fas (eBioscience/Affymetrix).
3. TNF-α (eBioscience/Affymetrix).
4. Staurosporine (Sigma).
5. Actinomycin D (Sigma).
6. Etoposide (Sigma).
7. Camptothecin (Sigma).
8. Cycloheximide (Sigma).
9. Fluorescein-labeled Annexin V (eBioscience/Affymetrix).
10. Propidium Iodide (Life Technologies).
11. Flow cytometer.

2.2 Analysis of the Intracellular Role of Galectins in Apoptosis Using Primary Cells from Galectin-Deficient Mice

2.2.1 Isolation and Culture of Primary Macrophages from Galectin-3 Knockout Mice

1. Galectin-3 KO mice (*see* Note 4).
2. C57BL/6 wt mice.
3. Brewer’s thioglycollate broth (Difco Laboratories).
4. 10 ml syringe.
5. 25 gauge needles.
6. 18 gauge needle.
7. PBS, 1 mM EDTA.
8. Clinical centrifuge.
9. Media: DMEM supplemented with 10 % FBS.

[4]Galectin-3 KO mice on a C57BL/6 or BALB/c background are distributed worldwide and available from the author’s laboratory on request.

2.2.2 Induction and Measurement of Apoptosis in Activated Macrophages

1. 5 ml Teflon beaker (Thermo Fisher).
2. IFNγ (Roche Biochemicals).
3. DMEM supplemented with 10 % FBS.
4. *E. coli* lipopolysaccharide (LIST Biologicals).
5. Fluorescein-labeled Annexin V (eBioscience/Affymetrix).
6. Propidium Iodide (Life Technologies).

2.2.3 Isolation and Culture of Primary Keratinocytes from Galectin-3 Knockout Mice

1. Neonatal wild-type and galectin-3 KO C57BL/6 mice (12–48 h after birth).
2. 70 % ethanol.
3. 100 mm Petri culture dishes.
4. Small scissors.
5. Curved scissors.
6. Blunt forceps.
7. Curved forceps.
8. Surgical scalpel handle.
9. Sterile surgical blades.
10. Dispase II (Roche Biochemicals).
11. Epilife medium (Invitrogen).
12. Supplemented Epilife Medium: Epilife Medium supplemented with 0.06 mM $CaCl_2$, 10 ng/ml mouse epidermal growth factor, 10^{-10} M cholera toxin, and human keratinocyte growth supplement-V2.
13. Phosphate-buffered saline.

2.2.4 Induction and Measurement of UV-Induced Apoptosis in Keratinocytes

1. Phosphate-buffered saline.
2. 10 cm dishes.
3. UV crosslinker (Stratagene/Agilent) fitted with BLE-8T312 UVB lamps (Spectronics Corp.).
4. UVB-Meter model UVB-500C (National Biological Corporation).
5. CO_2 incubator.

2.2.5 Treatment with Hydrogen Peroxide and Etoposide to Induce Apoptosis

1. Hydrogen peroxide.
2. Etoposide.
3. CO_2 incubator.

2.2.6 Detection of Apoptosis by Flow Cytometry

1. 100 mm Tissue culture plates.
2. Trypsin 0.25 %.
3. EDTA 0.1 %.
4. Trypsin and trypsin inhibitor (Cascade Biologics/Invitrogen).
5. Fluorescein-labeled Annexin V (eBioscience/Affymetrix).
6. Propidium Iodide (Life Technologies).
7. F FACS Calibur (Becton Dickinson) or comparable flow cytometer.

2.2.7 Detection of Apoptosis by Nuclear Staining with Hoechst 33342

1. Lab Tek Chamber Slide (Thermo Scientific Nunc).
2. Phosphate-buffered Saline.
3. 4 % paraformaldehyde.
4. Hoechst 33342.
5. Fluoromount-G (Southern Biotech).
6. Cover slips.
7. Fluorescence microscope.

2.2.8 Detection of Apoptosis by Quantitative Estimation of Histone-Associated DNA Fragmentation by ELISA

1. Flat-bottomed six-well plates (Falcon, Becton Dickinson).
2. ELISA Kit (Cell Detection ELISAPLUS, Roche Diagnostics Co.).
3. Cell Detection ELISA^{PLUS} Kit (Roche Biochemicals).
4. ELISA Microplate Reader.

3 Methods

3.1 Analysis of the Intracellular Role of Galectins in Cell Growth and Apoptosis by Altering Gene Expression Levels in Cell Lines

3.1.1 Examination of the Role of Galectin-3 Expression in Jurkat E6-1 Cells (See Note 5)

1. Precipitate linearized plasmid DNA with ethanol in the presence of 0.25 M NaCl.

2. Add 0.5 ml 70 % ethanol and rinse the internal surface of the tube.
3. Aspirate ethanol under sterile conditions in a laminar flow hood and briefly air-dry for 5 min.
4. Resuspend DNA in sterile TE at 2 mg/ml (*see* Note 6).
5. Spin down 1–2 × 10^6 actively proliferating cells at 1,100 rpm (280 × *g*) for 5 min at 4 °C, and decant supernatant (*see* Note 7).
6. Resuspend cells in 0.4 ml complete culture medium. Transfer cells to a 0.4 cm cuvette and add 15 μl of DNA (2 mg/ml).
7. Electroporate with BioRad Gene Pulser with Capacitance Extender, no pulse extender, at 260 V, 1,000 μF.
8. Transfer cells to 5 ml medium and return to the incubator.
9. Initiate drug selection 1 or 2 days after transfection with 1 mg/ml G418 (*see* Note 8).
10. Replace selection medium on alternating days in order to establish growth of transfected cells.
11. Continue cultivation with selection pressure for 2 weeks, expanding the culture as necessary, and freeze cell aliquots periodically after the 2-week period post-electroporation.
12. To determine galectin-3 expression from successful cell transfectants by immunoblotting, wash cells in PBS and add lysis buffer at 10^7 cells/ml on ice.
13. Vortex well, then return to ice, and repeat two times.
14. Microcentrifuge at >13,000 × *g*, 15 min, 4 °C, collect supernatant, and determine protein concentration by the BCA assay.
15. Run 20 μg extract on 12 % PAGE, transfer to a PVDF membrane (e.g., Immobilon P, Millipore), and process for detection with galectin-3 antibody.

3.1.2 Role of Galectin-3 by RNAi Suppression in HeLa Cells (See Note 9)

1. Resuspend Stealth RNAi siRNA duplexes in RNase-free water to make a 20 μM solution in 10 mM Tris–HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA.

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[5] As the human T-cell line Jurkat clone E6-1 does not express endogenous galectin-3 this cell line can be used as recipient for ectopic galectin-3 expression. The cells can be transfected by electroporation with a protocol generally applicable to a wide range of cell lines without pre-optimization [37].

[6] It is critical that the DNA for transfection is of high quality and purity. DNA is traditionally prepared by CsCl (cesium chloride) centrifugation. A number of reagent kits are commercially available for this purpose, such as Qiagen Plasmid Kits for various scales of plasmid DNA purification. We routinely use a cost-efficient method involving alkaline lysis, phenol extraction, and polyethylene glycol precipitation with satisfactory results [38, 39].

[7] The proliferative state of the cells greatly affects the transfection efficiency. We usually dilute near-saturated culture 1:3 with fresh growth medium the day before transfection to make sure that they are actively proliferating on the day of transfection.

[8] We use 1 mg/ml G418 for selection of Jurkat cells transfected with constructs carrying a neo-resistance cassette. Conditions need to be empirically determined for other selection markers and other cell lines.

[9] The human cervical cancer cell line HeLa expresses galectin-3 and can be used as a model to test the intracellular role of galectin-3 by suppressing endogenous galectin-3 using RNAi.

2. Transfect 2 pmol of siRNA into HeLa cells in a 24-well plate with Effectene reagent.

3.1.3 Assays of Cell Growth and Apoptosis in Cell Lines

1. Cells are assessed for cell survival under suboptimal conditions in low-serum medium (10^5 cells/ml in RPMI 1640/1 % FBS for Jurkat E6-1, or 10^5 cells in 3 ml DMEM/1 % FBS in a 6-well plate for HeLa cells).
2. Induction of apoptosis in Jurkat cells can be performed by the following methods:
   a. Incubation with 50 ng/ml anti-human Fas (eBioscience/Affymetrix) for 12–24 h.
   b. Overnight culture with 0.5 μM of staurosporine (Sigma).
3. Induction of apoptosis in HeLa cells can be performed by culturing 10^5 cells/well in 12-well dishes followed by overnight treatment with any of the following: actinomycin D (1 μM), etoposide (50 μM), camptothecin (1 μM), or a combination of TNF-α (10 ng/ml) and cycloheximide (10 μg/ml).
4. Surviving cells are determined by the MTS assay as previously described [35] (*see* Note 10).
5. Alternatively, early and late apoptosis can be differentiated by flow cytometry by detection of externalized phosphatidylserine and compromised membrane integrity by incubation with fluorescein-labeled Annexin V (10 μl/10^5 cells) and propidium iodide (PI, 1 μg/ml), and analyzed by flow cytometry in FL1 and FL3 channels, respectively [36].

3.2 Analysis of the Intracellular Role of Galectins in Apoptosis Using Primary Cells from Galectin-Deficient Mice

3.2.1 Isolation and Culture of Primary Macrophages from Galectin-3 Knockout Mice

1. Inject 3 ml thioglycollate broth using a 25 gauge needle into the peritoneum of each mouse, penetrating at the left lower quadrant [1] (*see* Note 11).
2. Three days later, wipe the abdomen well with alcohol, and then perform peritoneal lavage by injecting 10 ml ice-cold PBS/1 mM EDTA using a 10 ml syringe fitted with an 18 gauge needle into the peritoneum through the lower left quadrant with bevel side down (*see* Note 12).
3. Slowly begin to aspirate by withdrawing the syringe plunger while tenting the abdominal skin with the needle to avoid obstructing the needle opening.

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[10] The MTS method is similar to the MTT assay but avoids the need for solubilization with solvent and detergent.
[11] Estimate the numbers of mice required by calculating approximate yields of 8×10^6 macrophages/mouse.
[12] While the needle is inserted into the peritoneal cavity, gently roll (vigorous rolling may cause leakage of fluid from the highly distended abdomen) the mouse side to side with the needle in order to gently disturb the fluid within the peritoneum while maintaining a good needle seal against the mouse skin so as to prevent fluid leakage. This can increase cell yields. Avoid downward orientation of the tip of the needle in order to prevent rupturing the intestines or causing bleeding.

4. Centrifuge the aspirate at 200 × *g* for 10 min at 4 °C.
5. Wash once in cold PBS.
6. Enumerate cells to determine leukocyte concentration.
7. Centrifuge required number of cells needed for the assay and resuspend cells at 2 × 10^6 cells/ml DMEM/10 % FBS.

3.2.2 Induction and Measurement of Apoptosis in Activated Macrophages

1. Culture 2 × 10^6 macrophages in a 5 ml Teflon beaker.
2. Add an equal volume of IFNγ from 0 to 50 U/ml and culture in CO_2 incubator for 6 h.
3. Recover cells by gently pipetting around the beaker to dislodge cells.
4. Centrifuge cells at 200 × *g* for 10 min at 4 °C.
5. Wash once in DMEM supplemented with 10 % FBS.
6. Reculture cells in Teflon beakers at 10^6 cells/ml in DMEM supplemented with 10 % FBS and 1 μg/ml *E. coli* lipopolysaccharide for 3 days.
7. Harvest by gentle pipetting to dislodge macrophages (lightly adhered), and remove one-fourth culture daily for detection of apoptosis.
8. Cool cells and centrifuge at 4 °C.
9. Resuspend in annexin V staining buffer, add 10 μl FITC-annexin V per 10^6 cells, and incubate for 15 min.
10. Prior to analysis, add propidium iodide to 1 μg/ml and analyze for FITC and PI staining as described above.

3.2.3 Isolation and Culture of Primary Keratinocytes from Galectin-3 Knockout Mice

1. Euthanize neonatal wild-type and galectin-3 KO C57BL/6 mice (12–48 h after birth) by CO_2 inhalation from a tank of compressed gas.
2. Rinse them in 70 % ethanol.
3. Move the pups onto the cover of one 100 mm sterile plate.
4. Amputate limbs and tails with small sharp scissors.
5. Slit along vertical (mid) section from nose to tail.
6. Gently peel off the entire skin; avoid damaging the skin.
7. Flatten skins with the dermis side down on 10 mg/ml of Dispase II in Epilife medium at 4 °C overnight.
8. Separate epidermis from the dermis.
9. Mince the epidermis with sterile curved scissors.

10. Shake 50 times in medium and then discard large pieces.
11. Centrifuge at 200 × *g* for 5 min, and resuspend the cells in supplemented Epilife medium.
12. Plate the cells at a density of 5×10^4 cells/cm^2.
13. Incubate the cells at 37 °C, 5 % CO_2, for 48 h undisturbed, and then gently add additional supplemented Epilife medium to the plates.
14. Mouse epidermal keratinocyte foci can be seen after 3 days.
15. Change medium every 2–3 days.

3.2.4 Induction and Measurement of UV-Induced Apoptosis in Keratinocytes

1. Seed wild-type and galectin-3 KO mouse keratinocytes at a semi-confluent density, 24 h before irradiation.
2. Incubate the cells at 37 °C, 5 % CO_2, for 24 h.
3. Wash cells with PBS and add 5 ml PBS to cover cells (for 10 cm dish).
4. Irradiate cells with a dosage of 100–200 J/m^2.
5. Reconstitute the cells into fresh culture medium. Incubate the cells at 37 °C, 5 % CO_2.
6. Assay for cell death 8–24 h later.

3.2.5 Treatment with Hydrogen Peroxide and Etoposide to Induce Apoptosis

1. Seed wild-type and galectin-3 KO mouse keratinocytes at a semi-confluent density, 24 h before treatment.
2. Incubate the cells at 37 °C, 5 % CO_2.
3. Reconstitute with fresh culture medium.
4. Culture the cells with hydrogen peroxide (25 μM) or etoposide (25 μM) at 37 °C, 5 % CO_2, for 24 h.

3.2.6 Detection of Apoptosis by Flow Cytometry

1. Culture wild-type and galectin-3 KO mouse keratinocytes in 100 mm tissue culture plates.
2. Induce apoptosis with UVB irradiation, or hydrogen peroxide or etoposide treatment.
3. Twenty-four hours after induction of apoptosis, harvest both detached and attached cells.
4. Use mild trypsinization to collect the attached cells.
5. Neutralize with defined trypsin inhibitor.
6. Cool on ice, and then centrifuge the cells at 200 × *g* for 5 min.

7. Wash once with cold PBS.
8. Stain the cells with FITC-conjugated annexin V and propidium iodide (1 μg/ml).
9. Examine cells by two-color analysis using FACS Calibur in FL1 and FL3 channels, respectively.

3.2.7 Detection of Apoptosis by Nuclear Staining with Hoechst 33342

1. Plate wild-type and galectin-3 KO mouse keratinocytes on Lab Tek Chamber Slides.
2. Induce apoptosis with UVB irradiation, hydrogen peroxide, or etoposide treatment.
3. Twenty-four hours after induction of apoptosis, wash cells with PBS.
4. Fix the cells with 4 % paraformaldehyde.
5. Incubate for 10 min with Hoechst 33342 (5 μg/ml) in the dark at room temperature.
6. Wash with PBS.
7. Mount cover slips with Fluoromount-G.
8. Identify the morphological characteristics of apoptotic cells with the aid of a fluorescence microscope using the excitation wavelength of 340 nm.
9. Cells with fragmented and/or condensed nuclei are considered as apoptotic cells.

3.2.8 Detection of Apoptosis by Quantitative Estimation of Histone-Associated DNA Fragmentation by ELISA

1. Plate wild-type and galectin-3 KO mouse keratinocytes in flat-bottomed six-well plates.
2. Induce apoptosis with UVB irradiation, hydrogen peroxide, or etoposide treatment.
3. Twenty-four hours later, centrifuge the plates at 200 × *g* for 10 min.
4. Lyse the cell pellets in buffer provided with the ELISA Kit (Cell Detection ELISAPLUS, Roche Diagnostics Co.).
5. Measure soluble histone–DNA complex in the lysates as a result of DNA fragmentation by ELISA. Absorbance at 405 nm (reference at 492 nm) is measured in each well.

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