

miRNA Cell Tracer: Multifunctional Microgels for Spatially Resolved and Wide-Range Detection of Intracellular miRNA at Single-Cell Level

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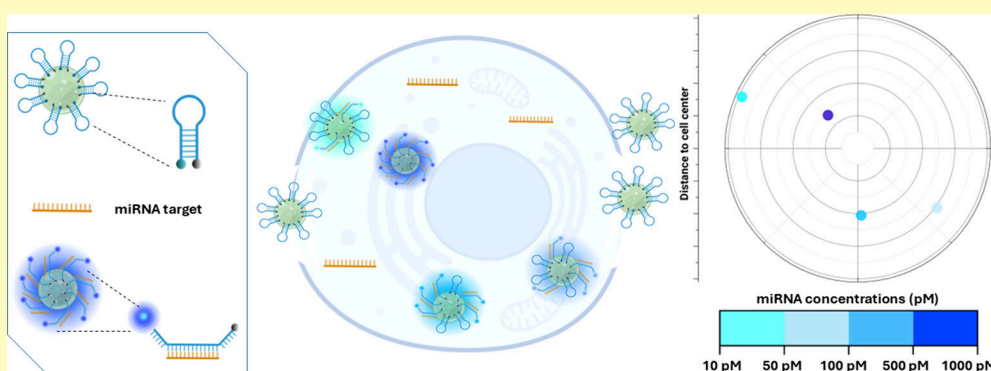


Supporting Information

ABSTRACT: Sensitive and spatially resolved detection of intracellular microRNAs (miRNAs) is essential for understanding gene regulatory networks at the single-cell level, yet low abundance and heterogeneous distribution challenge conventional methods. Despite the availability of a variety of particle biosensors, their intracellular use for live-cell miRNA detection remains challenging. Here, we

demonstrate an enabling amplification-free detection and spatial mapping of miRNAs in single cells. We employ large (~600 nm) biocompatible microgels, which provide high local probe density, protect molecular beacons from nuclease degradation, and enhance fluorescence signals upon hybridization with target miRNAs. Microfluidic mechanoporation efficiently delivers the microgels directly into the cytosol without perturbing endogenous miRNA levels or cell viability. Functionalized microgels generate robust fluorescence signals upon hybridization with target miRNAs. In intracellular sensing, the system achieves a low-picomolar limit of detection (5.6 pM) and a dynamic range spanning three orders of magnitude (pM–nM). The biosensor also exhibits excellent specificity; negligible fluorescence was observed both with nontarget and precursor miRNAs. Application in healthy (MCF10A) and cancer (MCF7) breast cells enabled single-cell quantification of endogenous miR-191-5p and miR-363-5p, revealing variability both among individual cells and between cell types, along with distinct intracellular localization patterns reflecting multiple concentration levels. The approach combines high sensitivity, wide dynamic range, quantitative precision, and spatial resolution, allowing amplification-free monitoring of miRNA expression in live cells. This versatile platform provides a powerful tool for intracellular biosensing, with potential applications in live-cell diagnostics, therapeutic response profiling, and studies of single-cell gene regulation.

KEYWORDS: microRNA detection, microgel biosensors, intracellular detection, single-cell analysis, spatially-resolved quantification, amplification-free optical detection, live-cell bioassay, mechanoporation delivery



Detecting intracellular DNA, RNA, and proteins provides valuable insights into the regulatory mechanisms governing physiological and pathological cellular processes.^{1,2} Among these molecules, microRNAs (miRNAs) play a crucial role as important biomarkers for studying cellular states in health and disease.³ However, the small size and low abundance of miRNAs present significant challenges for their detection and quantification, making conventional techniques inadequate,⁴ particularly for dynamic spatiotemporal quantification in living cells.^{5,6} Traditional miRNA detection methods like qRT-PCR, microarrays, and fluorescence-based techniques (e.g., FISH) have key limitations in live-cell applications. qRT-PCR and microarrays, though precise, require cell lysis, limiting analysis to a single time point and risking contamination from pri-/pre-miRNAs.

Live-cell FISH uses small or modified probes to improve cell entry but faces signal stability and specificity challenges due to probe accumulation in organelles.^{7–11} Additionally, in single-cell FISH, abundant signals can overshadow weaker ones, limiting the dynamic range and the accurate quantification of miRNAs in a single cell.¹² These limitations highlight the need for advanced

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techniques enabling real-time, accurate miRNA detection in live cells with high sensitivity and specificity. Recently, new in vitro miRNA detection methods have emerged, using both enzymatic amplification and nonenzymatic approaches.^{13,14} These methods offer good sensitivity but involve complex, multistep processes with cascade hybridizations, longer reaction times, and higher chances of nonspecific binding, which increases the risk of errors due to their multimolecular nature.^{15–18} A unimolecular amplification strategy can streamline detection by shortening reaction times, reducing nonspecific interactions, and enabling real-time monitoring. Nanoparticles and microparticles support this by offering high surface area for functionalization and tunable properties for stability and biocompatibility. However, challenges like low sensitivity and catalytic efficiency still hinder their consistent performance.¹⁹ Nanoparticles used for intracellular delivery often face limitations in sensitivity, response time, and signal transduction due to their small size and the complexity of the intracellular environment.^{20–25} The performance of gold nanoparticles (AuNPs), mesoporous silica nanoparticles (MSNs), lipid-based nanoparticles (LNPs), and polymeric microgels is closely linked to their sizes (10–300 nm). Smaller particles tend to aggregate, show low catalytic efficiency, experience steric hindrance at receptor sites, or localize incorrectly in cells.^{26–28} For example, AuNP-loaded split-DNAzyme probes reached 10 pM sensitivity in vitro, while carbon nitride nanosheet (CNNS)-based systems with catalyzed hairpin assembly (CHA) achieved 0.10 pM but required complex amplification steps.²⁸ Other materials, like oxides, semiconductors, or TiO₂, show similar challenges in sensitivity and fabrication. Despite promising femtomolar detection, improvements are still needed to boost sensitivity, specificity, and speed in live-cell applications.^{29,30} Small nanoparticles are less stable, prone to aggregation, harder to manipulate, and can diffuse rapidly, complicating target interactions and potentially entering organelles. Their limited probe loading and steric hindrance reduce signal strength and sensitivity.^{31,32} In contrast, larger nanoparticles such as polymer dots (Pdots) incorporate more chromophores, offering stronger fluorescence, better visibility, and improved detection limits for intracellular imaging.^{33,34} A major challenge for nanoscale biosensors is balancing response time with detection limits, both influenced by nanoparticle size through reaction transport kinetics and signal transduction efficiency.³⁵ Signal transduction determines the analyte amount needed for a measurable response, while transport kinetics relates to capture time. Since the critical concentration is inversely proportional to sensor surface area, larger sensors offer more binding sites, improving the chance of target capture even at low concentrations. This enhances sensitivity and enables trace analyte detection with greater precision. Our group has developed microgels—in a wide range of dimension from 600 to 1000 nm—for miRNA quantification at femtomolar concentrations via a unimolecular binding reaction, with a broad detection range (10 fM–10 nM).^{36–38} These microgels address limitations of smaller particles, enhancing intracellular detection by increasing signal density and reducing background noise.^{36–38} While larger particles improve fluorescence intensity and visualization, their cellular uptake is, however, less efficient than that of smaller nanoparticles (10–300 nm), posing a trade-off between brightness and intracellular accessibility.^{39–43} Efforts to enhance cellular delivery include gene guns, microinjection, and lipofection, but each has limitations: low yield and cell viability (gene gun), invasiveness (microinjection), and inconsistent efficiency or toxicity (lipofection). Addressing these

issues is key to improving delivery efficiency, reducing cytotoxicity, and enhancing reproducibility.^{44–47} These factors highlight two interconnected needs: the use of large nanoparticles for highly sensitive, wide range, and reliable detection coupled with a system that ensures the efficient internalization of these large particles into living cells without compromising cell viability.

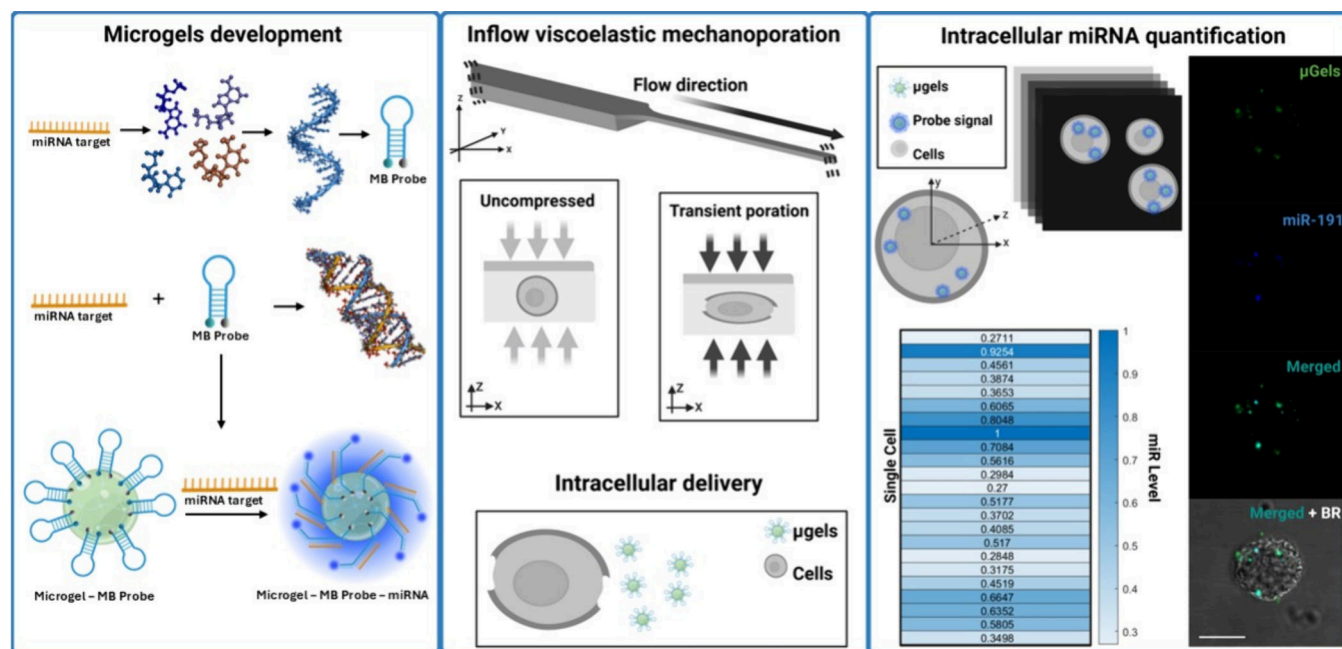
In this study, we present the development of unimolecular microgel-based biosensors for miRNA detection in living cells, miRNA cell tracer. The system works by utilizing 600 nm microgel particles conjugated with molecular beacon probes, which are internalized into cells through mechanoporation within a microfluidic platform (Scheme 1). Unlike conventional methods, miRNA cell tracer integrates molecular beacons directly onto the microgel surface, where their hairpin loop structure remains inactive until hybridization with the target miRNA, triggering a strong fluorescent signal upon binding. The high probe density on the microgel surface dramatically amplifies the signal, enabling exceptional sensitivity even for low-abundance miRNAs. Additionally, our laboratory has developed an advanced microfluidic platform for inflow cell mechanoporation, ensuring precise and highly efficient microgel delivery into cells without affecting viability.⁴⁸ This breakthrough guarantees reliable and reproducible miRNA quantification at the single-cell level, setting a new standard for miRNA detection inside living cells. As a proof of concept, this study focuses on two miRNAs with contrasting expression profiles in healthy (MCF10A) and low-invasive cancer (MCF7) breast cells: microRNA-191, which is highly expressed, and microRNA-363, which is effectively absent. The high expression of miR-191-5p and the absence of miR-363-5p in these cell lines are supported by publicly available RNA-seq datasets and database entries,^{49–51} which report abundant levels of miR-191-5p and negligible levels of miR-363-5p. By pairing a highly abundant miRNA with one that is absent, our platform can be evaluated at representative extremes of expression, confirming its reliability in detecting both strong and minimal signals.

We demonstrate that our system enables sensitive, wide-range, and three-dimensional (3D) spatially resolved quantification of miRNAs within cells, allowing precise tracking of their expression and distribution within the intracellular environment. This approach represents a significant advancement in biosensing technology, enabling efficient delivery and precise detection of miRNAs in complex intracellular environments at the single-cell level.

EXPERIMENTAL SECTION

Probe Design

To achieve high specificity in detecting miR-191-5p and miR-363-5p, the MB probes have been carefully designed based on alignment studies and thermodynamic parameter simulations. The specificity of the probe was assessed in silico using a Primer-BLAST tool (<https://blast.ncbi.nlm.nih.gov>), (Table S1). Thermodynamic parameters, including melting temperature, stability, the folded fraction at the dosage conditions, and the Gibbs free energy (ΔG) for MB hybridization to the targets, were calculated using appropriate tools like UNAFold web server and NUPACK. Simulations of the miRNA–target duplexes were performed using the Chemistry at Harvard Macromolecular Mechanics (CHARMM) software package. The simulations were carried out to analyze the potential energy components of the complexes, including bonded interactions such as bond stretching (BOND), angle bending (ANGLE), and torsional rotations (DIHEDRAL). The duplexes were solvated and simulated under physiological ionic conditions, specifically 0.15 M NaCl, KCl, and MgCl₂ at 37 °C and pH 7, to closely replicate the intracellular environment. These simulations provided

Scheme 1. Schematic Workflow of the Developed Strategy for Intracellular microRNA Detection Using Functionalized Microgels^a

^aMicrogels development: MB oligonucleotide sequences specific to target microRNAs are designed via computational analysis for predicting the nucleic acid secondary structure and hybridization thermodynamics and optimized using 3D simulations, to ensure high specificity and hybridization stability. The optimized MBs are then used to functionalize ~600 nm microgels (μGels). Intracellular delivery via inflow viscoelastic mechanoporation: Suspended cells are subjected to a highly viable and controlled mechanoporation procedure via microfluidics. Then, the functionalized μGels are delivered inside the cells. Intracellular microRNA quantification: Once internalized, μGels detect and quantify target microRNA levels based on fluorescence signal changes from the MB probes, enabling precise analysis of intracellular microRNA expressions.

detailed energetic profiles that informed the stability and structural behavior of the probe–target interactions (Table S2).

Molecular Beacon Fluorescence Assay

Fluorescence-based hybridization assays were performed for both molecular beacons targeting miR-191-5p and miR-363-5p. MBs (50 nM in TE buffer) were incubated with their respective target oligonucleotides at 1:1 or 1:2 molar ratios for 15 min at room temperature. miR-16, reported to be expressed in both MCF7 and MCF10A cells, was included as a nonspecific control.⁵² Fluorescence measurements were carried out using a Duetta Spectrofluorometer (Horiba) with fluorophore-specific excitation and emission settings. Reactions containing nontarget sequences served as additional specificity controls. Fluorescence intensities were compared to confirm selective hybridization of each MB to its complementary target (Figure S1).

Microgels Synthesis

Microgels labeled with the ATTO488 fluorophore were synthesized using free-radical pre-precipitation polymerization.³⁶ In summary, sub-micrometer particles were produced by mixing 1% (w/v) PEGDMA (poly(ethylene glycol) dimethacrylate), 0.034 mM acrylic acid, and 0.0094 mM ATTO488-maleimide dye in 1% (w/v) PVA (polyvinyl alcohol) with KPS (potassium persulfate) (2.2 mM) serving as the initiator, and the polymerization was carried out at a constant temperature of 65 °C for 6 h under a nitrogen atmosphere. Following polymerization, the microgels containing ATTO488 fluorophore were dialyzed for 15 days and purified multiple times via centrifugation (15 min at 6500 rpm) to eliminate any unreacted monomers, oligomers, and surfactants. The microgels were then stored at 4 °C until further use. Their size, zeta potential, conductivity, and electrophoretic mobility were analyzed using dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS instrument, 633 nm laser, 173° scattering angle). The

number of carboxylic groups present on the microgels was measured by potentiometric titration before the conjugation of the MB specific for miR-191-5p and miR-363-5p.

Scanning Electron Microscopy Analysis (SEM)

SEM was performed on a FE-SEM Ultra Plus (Zeiss) microscope at 20 kV. For sample preparation, the dialyzed microgels solution was fixed on a microscope slide, air-dried, and then sputtered with a 10 nm thin gold layer.

MB Bioconjugation to Microgels

MB probes with the fluorescent dye (ATTO647 N) to the 5'-end and a fluorescence quencher (BBQ-650) to the 3'-end were conjugated to microgels labeled with the fluorophore ATTO532. Bioconjugation was optimized by coupling 0.25 nmol of the MB. Briefly, 1 mg of microgels was incubated in 250 μL of 2-(N-morpholino)ethanesulfonic acid (MES) at pH 6, whereas MB was incubated in 50 μL MES at pH 6 for at least 5 h. Then, the carboxylic groups on the microgel were activated using 0.5 M of the coupling agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) by stirring vigorously briefly and leaving them on ice for 20 min. Subsequently, MB solution was added to the microgels by incubating overnight at 300 rpm at 4 °C. Microgels conjugated with MB were washed three times to remove the unreacted MB and suspended in 0.1 M TRIS (tris(hydroxymethyl)aminomethane). Unconjugated and conjugated microgels were characterized by DLS (dynamic light scattering). Briefly, the Z-potential and size of microgels with ATTO532 fluorophore, unconjugated and conjugated with 0.25 nmol of MB, were measured by dispersing microgels in H₂O, PBS (phosphate-buffered saline), or KCl (potassium chloride) 10⁻³ M at a concentration of 50 μg/mL. A total of three runs, each comprising three cycles, were conducted (Table S2), and results are the mean of the three runs. The efficiency of grafting was determined by comparing fluorescence measurements of the total MB added and the MB remaining in the

supernatant after conjugation, using a Horiba Duetta spectrofluorometer (counts/ μ A). Grafting efficiency was measured by relating the supernatant fluorescence value with respect to the total MB fluorescence.

Calibration Curve

For the final assay, 0.005 μ g/mL of microgels was used, conjugating 0.25 nmol of MB probe. The assay was performed by mixing the microgels (with ATT0532 fluorophore and conjugated with MB) with the target sequences, without preliminary steps of amplification. A calibration curve was done plotting the fluorescence of microgels over serial dilutions of synthetic miR-103-3p in TE. The samples were analyzed by confocal microscopy with an objective of 63 $\times$ 1.40 oil (Zeiss) using lasers at 543 and 633 nm. Laser power and detector gain were always kept constant with a scan speed of 8000 Hz. For each target concentration, 20 images were collected and analyzed. Images were processed and analyzed using ImageJ software. The assay for each concentration of miR-103-3p took about 1 h and was done in triplicate. Data are from 5 sets of 3 independent tests. To estimate LOD, 20 replicates of the blank sample (microgels without target) were measured.³⁹

Reverse Transcriptase and Quantitative Polymerase Chain Reaction (qRT-PCR)

For the qRT-PCR assay, 2 μ L of samples at varying concentrations was reverse transcribed using a TaqMan Advanced miRNA cDNA Synthesis Kit from Thermo Fisher Scientific. This involved extending the mature miRNA sequence through 3' poly-A tailing and 5' ligation of an adaptor sequence. Universal RT primers were employed to target the universal sequences at both the 5' and 3' ends of the mature miRNAs, converting all miRNAs in the sample to cDNA. Subsequently, 5 μ L of cDNA was utilized as a template for the TaqMan Advanced miRNA Assay, with each sample tested in triplicate. The TaqMan PCR protocol included an initial step at 95 $^{\circ}$ C for 20 s, followed by 40 cycles of 95 $^{\circ}$ C for 3 s and 60 $^{\circ}$ C for 30 s, conducted on a CFX-96 real-time thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A calibration curve was generated using serial dilutions of synthetic miR-191-5p and miR-363-5p in TE buffer.

Gold-Standard Quantification of Intracellular miR-191-5p and miR-363-5p

To evaluate the expression of miR-191-5p and miR-363-5p in MCF7 and MCF10A cells, a bioinformatic analysis was first conducted using RNA sequencing data from the public miRNA databases. Following this, experimental validation was performed on MCF7 and MCF10A cells using real-time PCR (qRT-PCR) before and after treatment. MCF7 cells were plated at a density of 20,000 cells/cm² in 75 cm² flasks and cultured in MEM medium containing 10% FBS (fetal bovine serum), 1% L-glutamine, and 2% penicillin-streptomycin (P/S). MCF10A cells were plated at a density of 20,000 cells/cm² in 75 cm² flasks and cultured in MEBM medium supplemented with a mammary epithelial growth media bullet kit (Lonza). Upon reaching 80–90% confluence ($\sim 1 \times 10^7$ cells), intracellular miRNAs were isolated using a mirVana miRNA Isolation Kit (Thermo Fisher Scientific), following the manufacturer's protocol. As a control for extraction efficiency, 1 μ L of 1 nM synthetic Cel-miR-39-3p was spiked into each sample prior to extraction. RNA concentration and purity were assessed using a Nanodrop 2000 spectrophotometer. Expression levels of miR-191-5p and miR-363-5p before and after electroporation in MCF7 cells (Figure S2) were normalized to the exogenous control Cel-miR-39-3p. Expression levels of miR-191-5p in MCF7 and MCF10A cells (Figure 3b) were normalized to the endogenous housekeeping miRNAs miR-93 and miR-16 to account for sample-to-sample variation.⁵³ Three biological replicates were tested, with each assay (for miR-191-5p and miR-363-5p) performed in triplicate. Data represent the mean of three technical replicates per sample.

MTT Assay

MCF7 and MCF10A cells were seeded in triplicate in 96-well plates at a density of 6000 cells/well and allowed to adhere overnight. Six hundred nanometer microgels were diluted in cell culture medium at the same final concentrations used for uptake experiments of control and deformed cells and added to the cells for 10 min and 1 h. After the incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used according to the manufacturer's instructions. Briefly, 200 μ L of MTT labeling reagent (final concentration 0.5 mg/mL; Sigma-Aldrich) was added to each well for 3 h. Then, the supernatant was removed and substituted with 500 μ L of isopropanol for 15 min at 37 $^{\circ}$ C, 5% CO₂. The optical density of each well sample was determined at 570 nm using a microplate reader. A blank absorbance value (0.09 and 0.11 for MCF7 and MCF10A, respectively), obtained from wells without cells but treated with cell culture medium and MTT reagent, was subtracted from all the absorbance values. Then, the average absorbance value of cells treated with microgels was normalized to that of control cells incubated with media and cell viability was expressed as a percentage of the control. Standard deviations of normalized absorbance values were calculated via error propagation.

Assessment of Microgel Stability in a Physiologically Relevant Intracellular-Like Environment

Cells were cultured to 90% confluence, washed with PBS, and detached using trypsin. Cells were collected by centrifugation (300 $\times$ g, 5 min) and washed twice with cold PBS. Approximately 10 million cells were used to prepare the lysate via six freeze–thaw cycles between dry ice and 37 $^{\circ}$ C, combined with vigorous pipetting for homogenization, as previously described.⁵⁴

To evaluate microgel stability and degradation kinetics, the microgels were incubated in the intracellular-like environment and observed by confocal fluorescence microscopy at 1, 3, 6, 12, 24, and 48 h. Fluorescence emission from both the microgel fluorophore (ATTO488) was used to assess particle integrity and stability (Figure S19). As proof of concept, extracts from MCF10A cells were used.

Experimental Setup for Inflow Cell Mechanoporation

The setup includes a pressure pump (P-pump, Dolomite Microfluidics), a round-shaped flexible fused silica capillary tubing (Molex), an ad-hoc designed microfluidic device, a fluid connector (N-333, IDEX), and an inverted microscope (X81, Olympus) with CMOS camera (ORCA FLASH 4.0, Hamamatsu Photonics K.K.). For the device fabrication, PDMS was employed owing to the simplicity of molding it in various shapes along with its inherent properties (transparency and biocompatibility). Briefly, a pressure pump pushes the sample volume through the capillary—where cells perfectly align—in the microfluidic device inlet, while at the end of the device a reservoir collects cells (Figure S7). Moreover, to induce compression and mechanoporation of single cells under viscoelastic flow conditions, we calibrated the initial pressure of ~ 1000 mbar for an applied flow rate of ~ 0.05 μ L/s. The used fluid was a highly biocompatible polyethylene oxide (4MDa, Sigma Aldrich) at a polymer concentration of 0.53 wt %.⁴⁸ We have investigated MCF10A, cultured in MEBM medium supplemented with the mammary epithelial growth media bullet kit (Lonza), and MCF-7, cultured in MEM medium containing 10% FBS, 1% L-glutamine, and 2% penicillin-streptomycin (P/S). For the inflow mechanoporation procedure, we start from cells diluted in 500 μ L of the viscoelastic medium to reach a final cell concentration of circa 50 cells per μ L. In a typical experiment duration of 60 s, ~ 3 μ L of cell suspension (concentrated to be 10⁶ cells/mL in the stock solution) is pushed through the chip and investigated by the imaging system, resulting in a total amount of ~ 150 cells/measurement. To assess cell viability, a Trypan blue test on the cell sample was performed both before and after mechanoporation.

Intracellular Delivery of Microgels

Before mechanoporation, cells were stained with Wheat germ agglutinin (WGA) used for colocalization analysis and incubated at room temperature for 30 min with a WGA solution at a final concentration of 1:200 μL . Green fluorescent microgels were delivered inside cells immediately after recovering from the microfluidic device. In detail, from the stock, microgels were added to culture medium at a final concentration of 1:100 μL . Then, 90 μL of this solution were added to 9 μL of treated cells to allow interaction between cells and gels. The resulting cell-to-particle ratio was 0.1. They were allowed to interact for 10 min inside the incubator. Time of interaction was chosen according to previous experiments.⁴⁸ These procedures were performed for all the analyzed experimental conditions: spontaneous delivery without mechanoporation and internalization after mechanoporation. After that, cells were fixed with 4% paraformaldehyde (PAF-Sigma-Aldrich) for 15 min at room temperature, then rinsed twice with PBS. This helped in the confocal acquisition. Moreover, since cells are in suspension, we performed a poly-L-lysine treatment onto μ -slide 8-well glass surfaces (Ibidi GmbH, Germany) to allow for cell interaction with the surface and stabilization after cell sedimentation.

Confocal Imaging and Analysis

The image stacks for the microgels intracellular delivery, colocalization, miRNA detection, and calibration analysis were collected at 0.35 μm Z-spacing using a 63 $\times$ oil immersion objective (NA 1.4) of a confocal laser scanning microscope (TCS STED CW, LEICA). Image analysis was performed using the open-source Fiji (ImageJ) program. For colocalization between inner cell membranes and delivered microgels, the overlapping areas between the red and green channels—for WGA and microgels, respectively—were quantified and presented as a percentage of not-colocalized microgels inside cells. In detail, we calculated the overlap by first thresholding each channel to define foreground pixels. Then, we determined the fraction of intersection of the two foreground areas compared to their union.⁵⁵ Instead, for colocalization and correlation evaluation of the microgels and miR-191-5p signals, we used a JacoP Plugin to obtain the output of both scatter plots and Pearson coefficients.⁵⁵ As a calibrating signal for the image analysis, miR-363-5p was investigated as follows: a region of interest (ROI) was identified around the single cell to be analyzed. Then, through the 'z-projection' function, the z-stack collapsed into a single image on which all the intracellular microgels and miRNA signals are contained. On the green channel, microgels were detected with individual ROIs—placing a threshold range from 40 to 255—useful for the miRNA localization. On the far-red channel, the ROI of microgels was added and miR-363-5p was measured. The same procedure of imaging investigation by ROIs was used for miR-191-5p. As reported from the histogram analysis of the collected integrated densities (Figure S11), the measured mean intensity (~ 6.5) was then applied as background signal of the miR-191-5p analysis (Figure S12). For calibration data, images were collected for different target concentration conditions. The limit of detection (LOD) was quantified referring to the first four concentrations (from 0 to 10 pM) and estimated as follows:⁵⁶

$$\text{LOD} = 2 \frac{\text{std deviation}}{\text{slope}}$$

where the std deviation stands for the standard deviation of the intensity data population collected for the zero concentration of target and the slope comes from the linear fit performed on the 0–10 pM concentration range. Statistical analysis for histogram plots, linear fitting, correlation investigation, and significance tests with Kruskal–Wallis nonparametric approach was performed using Microsoft Excel 2025 and Matlab2024b.

RESULTS AND DISCUSSION

Intracellular detection of microRNAs (miRNAs) remains a major analytical challenge due to their low abundance, dynamic

expression profiles, and heterogeneous spatial distribution.^{12,13} Because miRNA concentrations can vary dramatically with sub-cellular localization and cellular state,^{61–66} ranging from femtomolar to nanomolar levels, detection technologies must combine high sensitivity with a broad dynamic range to enable accurate quantification at the single-cell level. In this study, we integrate a rationally designed molecular beacon probe to functionalize biocompatible large-size microgels and to achieve high sensitivity, a wide range from subpicomolar to nanomolar concentrations, and 3D spatial resolution that enables direct visualization of intracellular miRNA gradients in live single cells.

Compared to traditional methods like qRT-PCR^{29,53} and FISH,^{8,9} our system offers several distinct advantages. First, unlike PCR, which relies on reverse transcription and amplification and needs cell lysis or RNA extraction, our approach eliminates these steps by directly detecting miRNAs inside the cells. This bypasses the need for enzyme-based reactions that depend on thermal cycling, thus avoiding sequence-specific biases, such as the denaturation of GC-rich regions that can compromise PCR data. Additionally, unlike FISH, which requires cell fixation and sample preparation, our system enables direct, in vivo detection without altering the native cellular state, providing more accurate, dynamic snapshots of miRNA expression.

This detection method provides an immediate and precise measure of miRNA levels within the cell, ensuring that the data accurately reflect the cellular state without the potential biases introduced by sample preprocessing due to gold-standard techniques.

Thermodynamic and Structural Characterization of miRNA–Probe Complex Stability

The minimum free energy (MFE) secondary structures of the designed probes exhibit good stability, with free energies of -5.44 and -6.66 kcal/mol (Figure 1a,b, left). Moreover, hybridization with their respective targets leads to a significant decrease in free energy, reaching -33.67 kcal/mol for the miR-191-5p complex and -32.14 kcal/mol for the miR-363-5p complex (Figure 1a,b, right), indicating strong thermodynamic stabilization. Molecular simulations conducted using CHARMM (Chemistry at Harvard Macromolecular Mechanics) confirm the formation of stable double-helical duplexes in the solvated 3D structures (Figure 1c,d). The detailed energetic data reported in Table S2 show that the miR-191-5p complex has the lowest total energy, reflecting a highly stable interaction. The miR-363-5p complex, while slightly higher in total energy, displays lower values for dihedral (DIHEDRAL) and improper (IMPR) energy components, suggesting reduced conformational strain. This greater structural flexibility may facilitate conformational adaptation during hybridization, improving binding efficiency with a lower energetic cost. Despite differences in their energetic profiles, both complexes exhibit high affinity and stability, making them promising candidates for use in high specificity biosensing applications.

Hybridization Probability and Specificity of the Probes

The hybridization probability of the probes has been further evaluated using base-pairing probability matrices for the folded probes (Figure 2a–c) and the hybridized probe to the target (Figure 2b–d). These matrices are based on thermodynamic equilibrium between the two RNA strands: the probe (Strand 1) and the target microRNA (Strand 2). As shown in Figure 2b–d, where the top-left and bottom-right quadrants represent the intrastrand secondary structures of the probe and target,

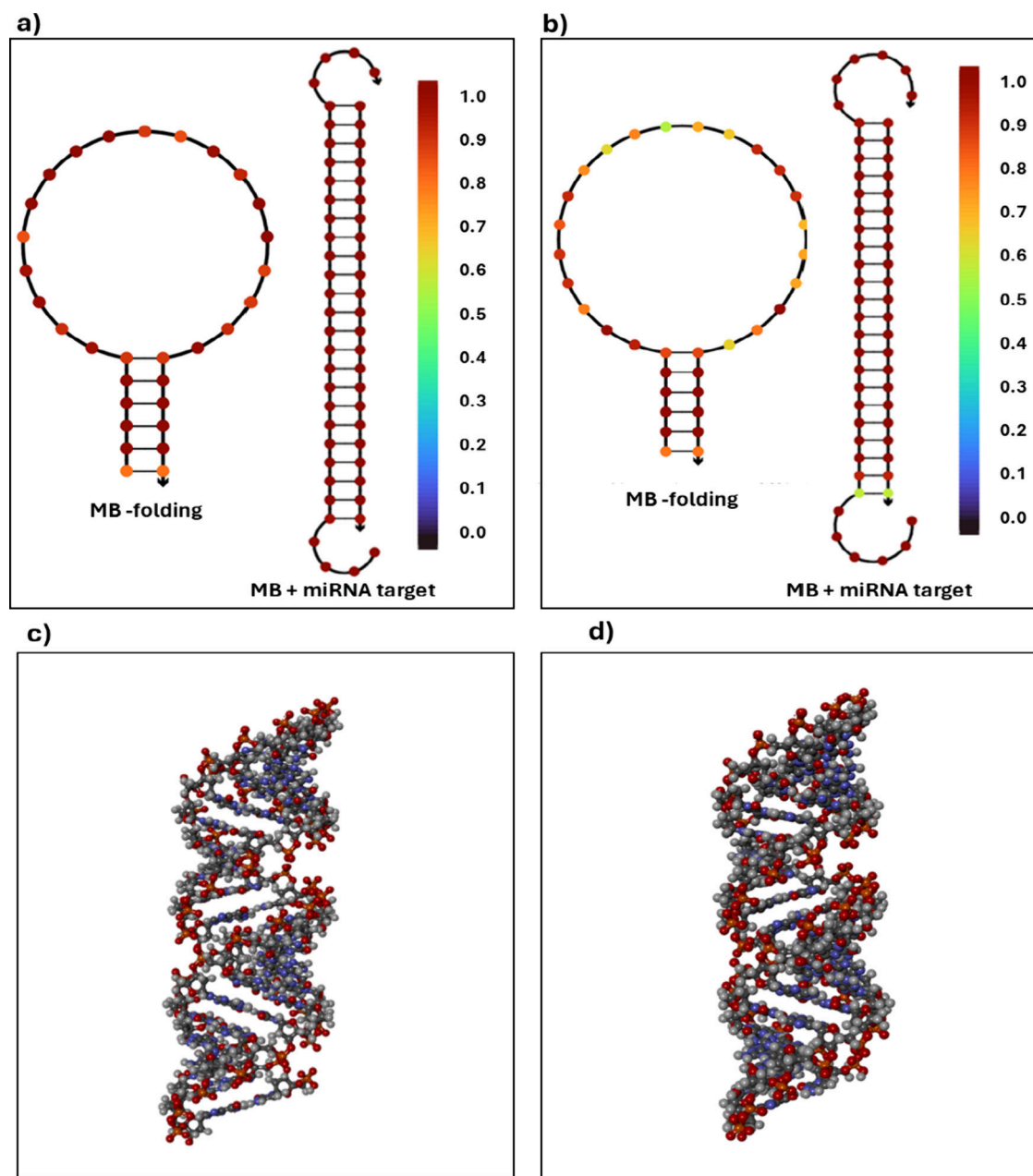


Figure 1. Secondary and three-dimensional structures of probes and probe-target complexes at 37 °C. (a, b, left) Minimum free energy (MFE) secondary structures of two hairpin probes, with free energies of -5.44 and -6.66 kcal/mol, respectively. (a, b, right) MFE secondary structures of the probe-target hybrid complexes, showing significantly lower free energies (-33.67 and -32.14 kcal/mol), indicating increased thermodynamic stability upon hybridization. Color scale represents base-pairing probability (blue = low; red = high). (a) miR-191-5p (b) miR-363-5p (c, d) Three-dimensional structures of probe-miRNA target duplexes, generated using 3dDNA and analyzed via molecular dynamics simulations in CHARMM. Ball-and-stick representations are shown with color-coded atoms: gray (C), red (O), blue (N), white (H), and orange (P). The solvated 3D structures confirm the formation of stable double-helical duplexes for each probe-target complex. Detailed energy components, including bonded and nonbonded interactions, are reported in Table S2 and demonstrate the high stability of the miRNA-target interactions; (c) miR-191-5p complex and (d) miR-363-5p complex.

respectively, while the top-right and bottom-left quadrants highlight interstrand base-pairing. The intense red coloring in the hybridization zones confirms the high probability of duplex formation between the probe and the miRNA, indicating strong affinity and specificity. This specificity was further confirmed through comparative analysis with other highly expressed miRNAs, choosing as proof MCF7 cell line, whose hybridization free energies and sequences are reported in Table S3. The

probe exhibits a significantly more favorable ΔG for miR-191-5p compared to other tested miRNAs. As shown in Figure S3, green-blue colors are indicative of a low probability of stable duplex formation with unspecific miRNA targets, while the secondary structure of the probe remains largely conserved. These data confirm that the probe design enables highly specific recognition of the miR-191-5p target while avoiding unwanted interactions with nontarget miRNAs. This in silico evidence was

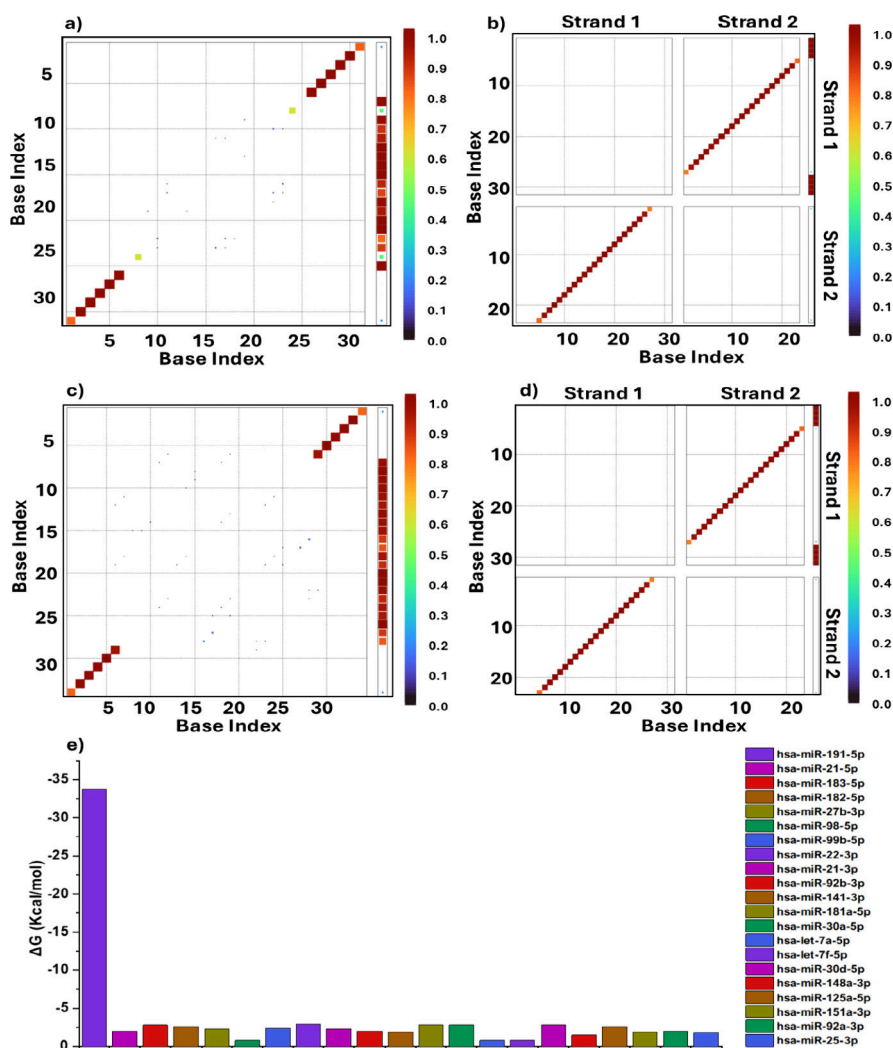


Figure 2. Base-pairing probability and hybridization specificity of probes for target miRNAs. The base-pairing probability matrices represent the equilibrium interactions between two strands: the designed probe (Strand 1) and the target microRNA (Strand 2). Panels (a) and (c) show the probability of intrastrand secondary structure formation within the probe, while panels (b) and (d) illustrate the interstrand hybridization probability between the probes and the targets. In all matrices, color intensity reflects the equilibrium probability of bond formation (ranging from 0 to 1, as indicated by the color scale). Red squares represent highly probable base pairings, while blue-green squares indicate progressively lower probabilities. (a, b) miR-191-5p and (c, d) miR-363-5p. (e) Bar graph of the free energy of hybridization (ΔG) between the probe for hsa-miR-191-5p, the specific microRNAs and nonspecific miRNAs (Table S3). The most favorable ΔG value (the most negative) corresponds to hybridization with miR-191-5p, indicating high specificity of the probe for its endogenous target compared to other abundant miRNAs.

further supported by a fluorescence hybridization assay, which confirmed the selective binding of the probes to their intended miRNA targets (Figure S1).

To rule out the possibility that the probe might also bind to precursor miRNA forms, we analyzed the predicted secondary structures of pre-miR-191-5p and pre-miR-363-5p, as shown in Figures S4 and S5. These precursor molecules exhibit stable folded conformations, with free energies of -46.38 kcal/mol for pre-miR-191-5p and -25.93 kcal/mol for pre-miR-363-5p, higher than those of probe folding conformation. The strong internal base-pairing within these precursors creates limited accessible single-stranded regions, thereby preventing effective hybridization by the probe. This structural constraint confirms that the microgel-based system is specific for mature miRNA detection and does not respond to pre-miRNA intermediates. These results demonstrate that our miRNA cell tracer system

effectively distinguishes mature miRNAs from their structurally similar precursors, overcoming the specificity limitations of conventional techniques such as FISH, as well as other hybridization-based methods like Northern blot and microarray.

Functionalization and Characterization of Microgels for Intracellular Delivery

The microgels were functionalized with MB through covalent bonding, with a grafting efficiency estimated at approximately 86%. Based on this, we estimate that each microgel contains around 2.1×10^4 MB molecules. Zeta potential analysis showed that the nonfunctionalized microgels possess a negative surface charge and a hydrodynamic diameter of approximately 600 nm (Table S4). After surface functionalization, the absolute value of the zeta potential decreased, reaching -1 mV, while the average particle size slightly increased to about 700 nm, indicating

successful attachment of functional groups and increased hydration of the surface layer. Although the zeta potential remains negative, the significant reduction in surface charge improves compatibility with the negatively charged cell membrane. This reduction in electrostatic repulsion facilitates the interaction between microgels and the plasma membrane, promoting intracellular uptake of the particles. These data suggest that functionalization not only modifies the surface composition but also actively modulates the colloidal properties of the microgels, enhancing their internalization efficiency in target cells. The material properties of microgels are central for the performance of the miRNA cell tracer. Their near-neutral zeta potential (~ -1 mV) reduces nonspecific interactions and protein adsorption, facilitating efficient cytoplasmic access while preserving intracellular signal stability. The structural design of the microgel ensures both efficient target molecule recognition and protection of probes from enzymatic degradation, enabling reproducible signal retention under physiological conditions.^{57,58} Surface functionalization, achieved via covalent conjugation of molecular beacons to the microgel surface, allows for a higher local concentration of oligonucleotide probes compared to embedding strategies, thereby increasing the sensitivity and responsiveness of the system. Furthermore, the covalent linkage stabilizes the probes, preventing their detachment and degradation by nucleases. This makes surface-functionalized microgels ideal for reliable and high-performance molecular detection inside the cells. Indeed, the SEM analyses show well-defined, spherical microgels that demonstrate excellent structural integrity. The particles are discrete and highly packed, confirming that their individual morphology was successfully preserved during the drying and imaging process. This uniformity is a key characteristic for effective applications (Figure S18).

To further assess the robustness of the system, microgel stability was evaluated in an intracellular-like environment over 48 h. Confocal fluorescence microscopy revealed that both the microgel fluorophore (ATTO488) and the molecular beacon probes fluorophore (ATTO647N) maintained their fluorescence and structural integrity throughout the incubation period (Figure S19), indicating minimal degradation or loss of function. Cell viability assays (MTT) performed on MCF7 and MCF10A cells confirmed that the microgels are fully biocompatible and noncytotoxic for exposures up to 1 h (Figures S16 and S17), further supporting their suitability for intracellular applications.

Intracellular Detection of miR-191-5p Using Functionalized Microgels

For the intracellular detection by using microgels inside MCF10A and MCF7, we verified that spontaneous and controlled delivery of these particles would not be possible without prior permeabilizing cells (Figure S6). For this reason, cells were subjected to a viable inflow mechanoporation after which microgels were passively delivered (Figures S7 and S8).⁴⁸ Of note, this possibility of using a mechanically homogeneous and noninvasive approach for transient membrane permeabilization further enhances the significance of this study. Indeed, unlike lipofection or electroporation, our mechanoporation approach avoids cytotoxicity and transfection variability while maintaining endogenous miRNA expression, as confirmed by qRT-PCR (Figure S2). Therefore, this method ensures physiological integrity that is an essential factor for accurate intracellular detection. In addition, despite the size greater than 500 nm, an absence of endocytic processes was assessed by observing no colocalization

between intracellular vesicles and microgels in both cell lines, with $\approx 80\%$ of free microgels inside cells (Figure 3a and Figure S9). A great optical accessibility of the miR-191-5p signal was then guaranteed, and the Pearson correlation coefficient quantified the degree of colocalization between microgels and detected target. For both MCF10A and MCF7, the average measured Pearson was between 0.75 and 0.85 (Figure 3b and Figure S10), indicating a good correlation between the two channels. Therefore, correct intracellular target molecule recognition and detection were allowed.

Figure 3a shows confocal images and 3D z-stack reconstruction of miR-191-5p signals inside MCF10A and MCF7, ensuring the well-defined distinct signals per cell. The images represent the z-stack reconstruction of cells, where microgels are labeled in green, miR-191-5p fluorescence in blue, and their colocalization in cyan. Quantitative analysis was displayed as a histogram of intensities revealing the distribution of miR-191-5p intensity detected in single microgels (Figure 3b). Each analyzed cell contained at least one microgel expressing a measurable miR-191-5p signal (Figure S7), demonstrating the capability of the system for single-particle intracellular miRNA detection even for different cell types. Moreover, all miR-191-5p intensities are greater than the background signal measured from the miR-363-5p quantification (Figures S11 and S12).

Validation of Sensitivity and Dynamic Range of the Detection System

The sensitivity and dynamic range of the system based on functionalized microgels for intracellular detection of miR-191-5p were evaluated by measuring fluorescence intensity as a function of target concentration. In detail, Figure 4a,b presents the confocal images of single microgels used as calibration standards, correlating fluorescence intensities to known picomolar (pM) concentrations of miR-191-5p (Figure S13). This also allowed determination of LOD of microgels, equivalent to 5.58 pM. Moreover, as shown in Figure S13, fluorescence intensity exhibits a clear correlation with miR-191-5p concentration in the picomolar (pM) range, with quantifiable response even at low concentrations. Log–log regression analysis reveals strong linearity ($R^2 > 0.98$), confirming the system's suitability for sensitive and wide-range quantification of endogenous miRNAs at low abundance. These results demonstrate not only the high sensitivity of the detection system but also its wide dynamic range, making the sensor effective for applications in realistic cellular contexts where miRNA concentrations can vary over several orders of magnitude.

Quantification and Intracellular Localization of miR-191-5p Using Functionalized Microgels

After validation of system sensitivity and LOD, we moved on to the quantification and localization of the intracellular pM miR-191-5p concentrations among different intracellular positions (Figure 4c). Then, considering the extracted linear relationship between fluorescence and pM concentration, we were able to determine the miR-191-5p quantities of interest, greater than the measured LOD. Both in MCF10A and MCF7, miR-191-5p concentrations resulted in being differentially distributed into the xy-plane. There, the positioning of microgels (Figure S14 and S15) shows a slight positive and negative correlation trend between distance and relative miR-191-5p expression for MCF10A and MCF7, respectively. However, these trends are not statistically significant.

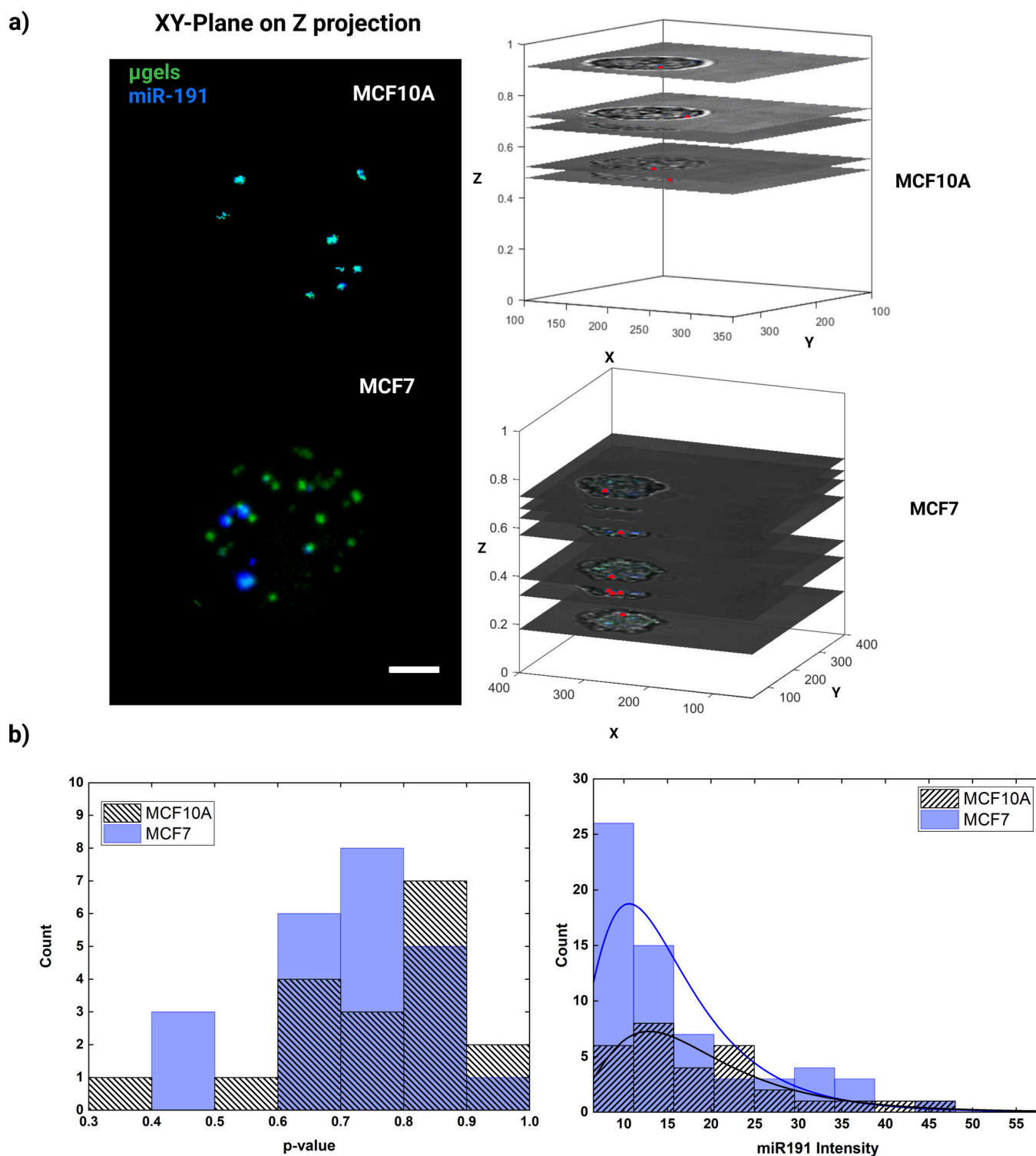


Figure 3. miR191 intracellular detection inside healthy and cancer breast cells using single microgels. (a) On the left, confocal stack reconstruction of MCF10A and MCF7 cells where the miR-191-5p-p signal was detected. On the right, localization of miR-191-5p in 3D z-stack and on the xy-plane, along z for MCF10A and MCF7. Scale bar: 10 μm . (b) On the left, the histogram distribution of p -values referred to the quality of colocalization between channels obtained for each cell line. On the right, the histogram distribution of intensities is presented. Number of analyzed cells: $N_{\text{Tot},\text{MCF10A}} = 15$ and $N_{\text{Tot},\text{MCF7}} = 23$.

To further strengthen the approach, a direct comparison with classical qRT-PCR analysis was performed (Figure 3 and Figure 4d). The two box plots of miR-191-5p intensities are not statistically different, like the qRT-PCR outcome, presented as a

column bar of mean and standard deviation. However, as expected for breast cancer cells,⁵⁹ a greater expression of miR-191-5p is detected in MCF7 than MCF10A, both for qRT-PCR and the proposed approach (Figures 3b and 4d), supporting both

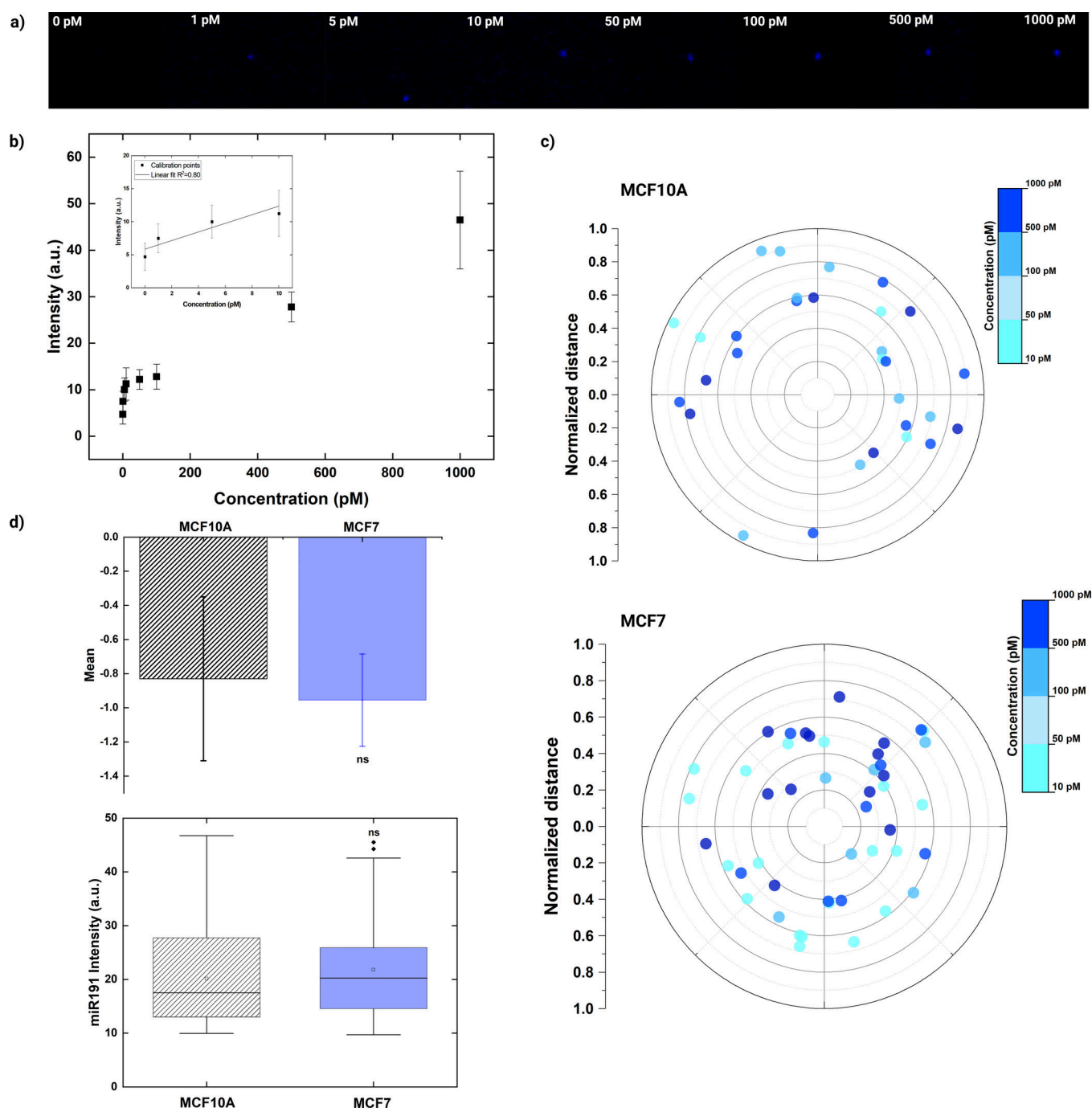


Figure 4. pM intracellular concentrations of miR-191-5p and their localization inside the cell. (a) Confocal images of single microgels used as calibrating objects for comparing the measured fluorescence intensities with the relative pM concentration of expected miR-191-5p. Scale bar: 10 μm. (b) Scatter data relating to how intensity signals change according to the target concentrations used. The inset plot contains the linear regression applied to the collected data in a dynamic range of concentration for LOD determination. (c) Polar plots of the different intracellular concentrations of measured miR-191-5p are shown to determine the relative positions of the detected concentrations on the xy-plane. (d) qRT-PCR results (on the top) and box plot of intensities (on the bottom) are shown, demonstrating a comparable expression and signal between the two lines. For each single cell, we measured the respective miR-191-5p intensities. Number of analyzed cells: $N_{\text{Tot,MCF10A}} = 18$ and $N_{\text{Tot,MCF7}} = 23$. ns: $p > 0.05$ the results are not statistically significant.

versatility and reliability with respect to gold-standard techniques. Moreover, in comparison with conventional bulk analyses, the approach enables a single-cell analysis.

In conclusion, the obtained spatial resolution capability is particularly valuable and powerful given the increasing evidence

that miRNA function is tightly linked to spatial context, affecting processes such as mRNA repression, cell migration, and metastasis.^{60,71} Furthermore, miRNA cell tracer reveals critical single-cell heterogeneity, uncovering wide cell-to-cell variability in miRNA expression that conventional bulk assays like qRT-PCR or

microarrays inherently mask. This level of resolution is indispensable in complex diseases such as cancer, where rare but pivotal cellular subpopulations can dictate therapeutic response, drive drug resistance, and fuel disease progression.^{67–70}

CONCLUSIONS

In this study, we have developed and validated a unimolecular microgel-based biosensor capable of detecting and spatially mapping intracellular miRNA concentrations with high sensitivity and specificity. Fluorescent-labeled polymeric microgels (600 nm), functionalized with molecular beacon probes, have been efficiently delivered into live MCF-10A and MCF7 cells via microfluidic mechanoporation, enabling amplification-free quantification of miR-191-5p with picomolar sensitivity over a broad dynamic range. The microgels retained favorable physicochemical properties after functionalization, including near-neutral zeta potential and structural integrity, which facilitated cellular uptake and minimized nonspecific interactions. miRNA cell tracer demonstrates high selectivity, accurately distinguishing mature miR-191-5p from structurally similar pre-miRNAs and nontarget sequences such as miR-363-5p, avoiding false positives. Beyond quantification, miRNA cell tracer allows spatial mapping of miRNA at the single-cell level, revealing a perinuclear enrichment gradient consistent with known miRNA maturation and export pathways, information not accessible through bulk or endpoint methods. Compared to conventional methods, our biosensor enables direct, non-destructive detection in live cells without amplification or lengthy hybridization. The unimolecular beacon architecture reduces background signals, enhancing specificity versus techniques like FISH, while material optimizations ensure biocompatibility and reliable performance. Moreover, with further optimization, this technology could be improved to become an effective tool for real-time monitoring of miRNA expression dynamics in living single cells, even of multiple types, without disrupting physiological processes. Therefore, this work establishes a robust and versatile approach for intracellular RNA detection, with potential applications in healthcare applications such as live-cell diagnostics, drug response profiling, and dynamic studies of gene regulation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.5c03434>.

Details about the used targets, sequences, and their own thermodynamics (Tables S1–S3); size and charge characterization of microgels (Table S4); figures describe RT-qPCR results and comparison between before and after the microfluidic mechanoporation for intracellular delivery with the relative sketch of used inflow approach; simulated probability of base pairing between targets and probes; histograms of miR-191-5p and miR-363-5p intensities inside cells; log–log linear fitting for LOD estimation (DOCX)

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Notes

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