

Near-Field Terahertz Nanoscopy Spatially Resolves Chiral Drug–Cell Interactions: Toward Precision Intracellular Pharmacology

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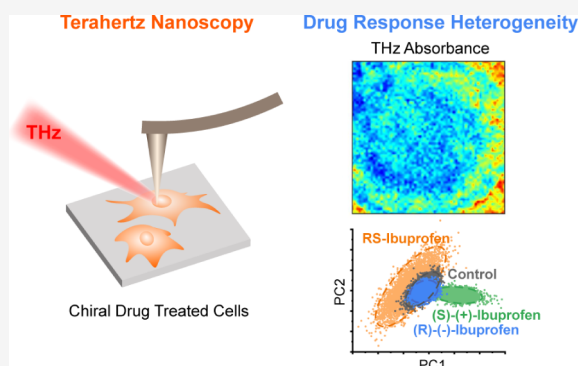
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ABSTRACT: Chiral drug enantiomers frequently exhibit marked differences in pharmacological efficacy and toxicity profiles. However, the resolution limitations of traditional detection methodologies render them incapable of resolving the spatial heterogeneity of drug distribution within individual cells and obscure our understanding of subcellular drug distribution and mechanism of action. Here, we employ terahertz scattering-type scanning near-field optical microscopy (THz s-SNOM), a cutting-edge technique offering nanoscale spatial resolution and label-free imaging capabilities, to investigate single-cell response heterogeneity to chiral drug exposure. We systematically analyzed morphological and biochemical compositional differences between control cells and those treated with RS-ibuprofen, (R)-(–)-ibuprofen, and (S)-(+)-ibuprofen. Our high-resolution analysis revealed that the mean area of plasma membrane-derived extracellular vesicles (PEVs) in drug-treated cells increased by 24.6%, 25.4%, and 39.9%, respectively, compared to the representative control cell. Through principal component analysis and Euclidean distance distribution methods, we identified distinct spectral variations in both intracellular and nuclear regions. Notably, these three enantiomeric forms induced fundamentally different patterns of heterogeneous evolution within cellular compartments. RS-ibuprofen treatment specifically resulted in increased membrane heterogeneity concomitant with decreased nuclear heterogeneity. Quantitative analysis of spectral intensity differences within nuclear regions ($p < 0.001$), based on single-frequency imaging and clustering analyses at characteristic THz peak frequencies, provided direct evidence of drug-induced heterogeneity. These findings establish a novel paradigm for mapping drug–cell interactions at the nanoscale, conceptualizing these processes as spatially encoded phenomena, thereby advancing the field of subcellular chiral pharmacology.



INTRODUCTION

Investigating drug responses at the single-cell level is essential for resolving cellular heterogeneity, a critical factor often obscured in bulk population analyses.^{1,2} This approach holds particular significance for chiral drugs, where enantiomer may exhibit distinct pharmacological, toxicological, and metabolic profiles due to stereoselective interactions with biological targets.³ Single-cell methodologies enable precise characterization of these differential effects, offering critical insights into drug efficacy, resistance pathways, and subcellular localization patterns that inform therapeutic optimization.⁴ While modern techniques like single-cell RNA sequencing and mass cytometry have revolutionized our understanding of cellular heterogeneity and drug-induced responses,⁵ they remain limited in spatial resolution for mapping subcellular drug distribution, which is a fundamental requirement for elucidating the mechanisms of action of chiral drugs.

Terahertz scattering-type scanning near-field optical microscopy (THz s-SNOM), which combines terahertz spectroscopy⁶ and near-field microscopy,⁷ addresses these limitations

through unique capabilities. Operating in the 0.1–10 THz range, this technology probes biomolecular vibrational and rotational modes (DNA, RNA, and lipids) for chemical fingerprinting while achieving nanometer spatial resolution. This combination gives THz s-SNOM unique advantages in cellular imaging studies, especially in label-free, high-resolution imaging and biomolecular identification.⁸ In recent years, the application of THz s-SNOM in cellular imaging has made remarkable progress.⁹ For example, Li et al. demonstrated THz near-field microscopy in the direct imaging of the morphology of single watermelon pulp cells in the natural dehydration process;¹⁰ Wang et al. reported a new THz near-field imaging method to observe the morphology and internal structure of

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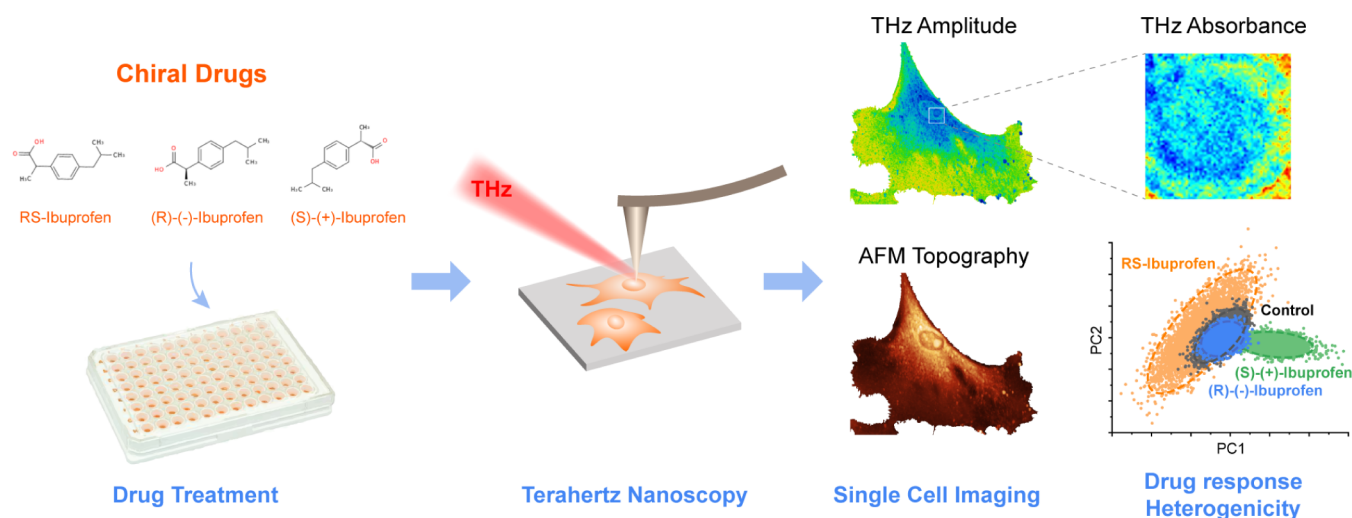


Figure 1. Schematic of THz s-SNOM detection of single cells treated with chiral drugs.

single bacteria.¹¹ Recently, we made a breakthrough in the application of THz s-SNOM for single-cell imaging, achieving an unprecedented spatial resolution of less than 80 nm.¹² This high resolution enabled the visualization of fine cellular structures, including membranes, intracellular organelles, and nuclear regions.¹² Importantly, we have also successfully extracted near-field absorption images at specific frequencies, allowing detailed visualization and compositional difference analysis. The ability to visualize and quantify biochemical changes at the nanoscale has far-reaching implications for fields such as pharmacology, opening up new avenues for studying intracellular heterogeneity and drug–cell interactions.

In this study, we employed THz s-SNOM to investigate single-cell responses to chiral drug exposure. Figure 1 illustrates the experimental schematic. Through the analysis of THz near-field amplitude images, we observed a significant increase in secreted plasma membrane-derived extracellular vesicles (PEVs) following treatment with three distinct chiral drugs. We conducted systematic characterization of drug-induced compositional variations in cellular components through principal component analysis (PCA) combined with the quantitative heterogeneity assessment of spectral data. Notably, our analysis revealed significant nuclear response heterogeneity variations among cells treated with different chiral drugs. By performing comparative absorption spectroscopy at multiple THz frequencies and conducting cluster analysis of single-frequency absorption images (captured at drug-specific characteristic resonance frequencies), we identified distinct nuclear compositional alterations corresponding to different drug treatments. These findings demonstrate the exceptional sensitivity of the THz s-SNOM in detecting nanoscale morphological modifications and biochemical reorganization induced by chiral pharmaceuticals. This work establishes a novel nanoimaging platform for investigating drug–cell interactions and tracking intracellular evolutionary pathways at the single-cell level, providing unprecedented spatial resolution beyond conventional analytical techniques.

MATERIALS AND METHODS

Stem Cell Preparation and Fixation. Primary human mesenchymal stem cells (hMSCs, obtained from iCell Bioscience Inc., Shanghai) were cultured in low-glucose DMEM (GIBCO) supplemented with 10% fetal bovine

serum (GIBCO), 100 U/mL penicillin, and 100 μ g/mL streptomycin. The cells were incubated under a humid atmosphere at 37 °C with 5% CO₂. Silicon wafers (1 cm $\times$ 1 cm), known for their broad biological compatibility, were used as the substrate. After being seeded onto the silicon wafers, the cells were treated with three different ibuprofen solutions: RS-ibuprofen, (R)-(-)-ibuprofen, and (S)-(+)-ibuprofen, each at a concentration of 1 mg/mL, for 2 h. Postincubation, the cells were gently rinsed three times with 0.01 M phosphate-buffered saline (PBS) and fixed with anhydrous ethanol for 20 min. The fixed cells were then washed 2–3 times with pure water and dried slowly under a stream of nitrogen gas. For the control group, cells seeded onto silicon wafers were directly processed without any additional incubation steps.

Cell Selection Criteria. An optical microscope (Zeiss Axio Imager.A2m) was used to check the uniformity of the cell distribution on the silicon wafer. Only those cells that were uniformly distributed on the silicon wafer and separated from other cells in the space were selected. Considering the size limit (100 μ m $\times$ 100 μ m) of the THz s-SNOM system, prolonged scanning time of cells that are too large will increase the risk of probe damage and may result in signal attenuation or distortion. Therefore, cells with extended cell membranes and sizes less than 300 μ m were finally selected for THz s-SNOM analysis. For each chiral drug-treated group, we collected and analyzed both AFM morphological data and THz near-field data from at least four individual cells. The cells in the same group exhibited similar spatial distribution patterns and THz spectral features, supporting the central conclusion that drug treatment induces distinguishable intracellular changes. To highlight the spatial analytical capability of the THz near-field imaging technique at the single-cell level and enhance the visualization effect, we selected one representative cell with clear morphological features from each group for presentation in our manuscript. Other cells were shown in Figures S1 and S2.

Near-Field Images of THz s-SNOM. A commercially available s-SNOM (THz s-SNOM, Neaspec GmbH, Germany) based on atomic force microscopy (AFM) was used under ambient conditions. The spectral range was 0.5–1.5 THz. Commercially available AFM tips (25PtIr200B, Rocky Mountain Nanotechnology, USA) with a tip radius of 40

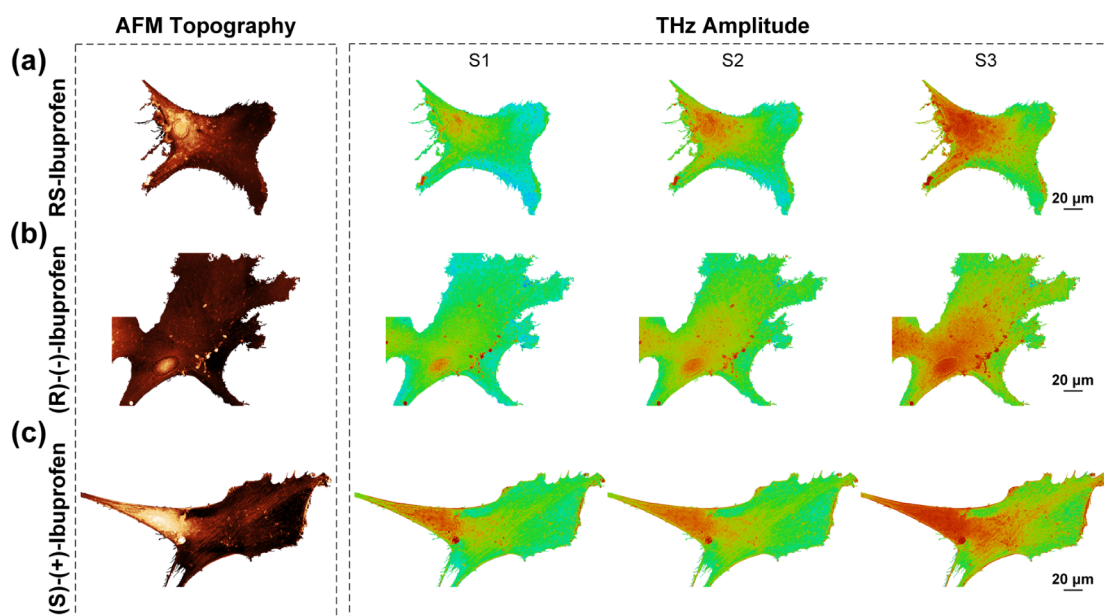


Figure 2. THz near-field imaging of representative cells treated with chiral drugs. (a)–(c) The atomic force microscopy (AFM) topographical images (left) and corresponding THz near-field amplitude images (right) acquired at the first (S1), second (S2), and third (S3) harmonic demodulation frequencies for cells treated with (a) racemic RS-ibuprofen, (b) (R)-(-)-ibuprofen, and (c) (S)-(+)-ibuprofen. Note the inverted color scheme for THz amplitude images in this figure (compared to subsequent figures) to enhance nuclear contrast. Scale bars: 20 μm .

nm, a tip length of 80 μm , a resonance frequency of about 50 kHz, and a vibration amplitude of about 200 nm were used. The probes were operated in the tapping mode to modulate the near-field scattered signal. A lock-in amplifier extracted the higher harmonics of the modulated signal, which were used as the THz near-field signal. The system obtained both AFM and THz near-field signals simultaneously. The AFM signal provided surface topography information, while the THz near-field signal contained both amplitude information and phase data. The AFM topographical images were processed using Gwyddion software (Version 2.58). Due to the maximum width of individual cells exceeding 100 μm , which exceeded the single-test limit of the instrument, near-field scanning was performed on separate regions of the cells. Complete AFM topographical images and THz near-field amplitude images for each cell were obtained using ImageJ software (Version 2.1.0).

Near-Field Spectroscopy of THz S-SNOM. Before the THz near-field absorption spectra were obtained, the AFM topographical images were scanned to identify the regions of interest. Each measurement was repeated four times at the same point, and the average values were used as the spectral data for that point. Data processing was performed by using Origin software. The THz near-field spectral data were smoothed using the Savitzky–Golay method (9 smoothing points). The sample spectra were normalized to the spectra of silicon wafers measured under the same environmental conditions. The THz near-field absorption spectra were obtained according to formula $a_n = \frac{S_n}{S_{\text{Si},n}} \sin(\varphi_n - \varphi_{\text{Si},n})$.¹³

MATLAB software was used to extract intensity values at specific frequencies from the absorption spectra of each pixel point. The THz near-field absorption images at specific frequencies for the region of interest were constructed. The absorption images were further normalized by using the corresponding height map to reduce the influence of

topography on absorption. Linear interpolation was applied to the output images.

PCA of THz Near-Field Absorption Spectra. The THz near-field absorption spectra were collected from the cells in each group (treated and untreated with chiral ibuprofen) in the intracellular and nuclear regions, respectively. To minimize spectral baseline differences, spectral data were preprocessed by normalization and centralization in MATLAB. PCA was then performed on the spectral data using the built-in PCA function in MATLAB. By calculating the covariance matrix and decomposing the eigenvalues, the principal components and their corresponding loadings were obtained. Based on the size of the eigenvalues, the first two principal components which accounted for more than 70% of the total variance were selected to construct score plots. The score plots highlighted the differences between the control and chiral drug-treated groups.

Heterogeneity Analysis of THz Near-Field Absorption Spectra. To quantify the heterogeneity difference in THz near-field absorption spectra of the drug-treated or untreated cells at intracellular and nuclear regions, the Euclidean distances between each of the two THz near-field absorption spectra were calculated according to the formula $D_{\text{Eu}}(p, q) = \sqrt{\sum_{i=1}^n (p_i - q_i)^2}$.¹⁴ Here, $D_{\text{Eu}}(p, q)$ was the Euclidean distance of the absorption spectra between two different scanning points, while p and q were n -dimensional vectors of the spectra of two different scanning points. Euclidean distance distributions of the spectra within a group reveal the inner-group heterogeneity; larger Euclidean distances indicate higher heterogeneities. In this study, heterogeneity comparisons of THz near-field absorption spectra of untreated and three chiral drug-treated cells were analyzed based on intracellular and nuclear regions, respectively. The heterogeneity distribution plots were constructed by Origin, which provides a comparative assessment of spectral variations induced by chiral drug treatment.

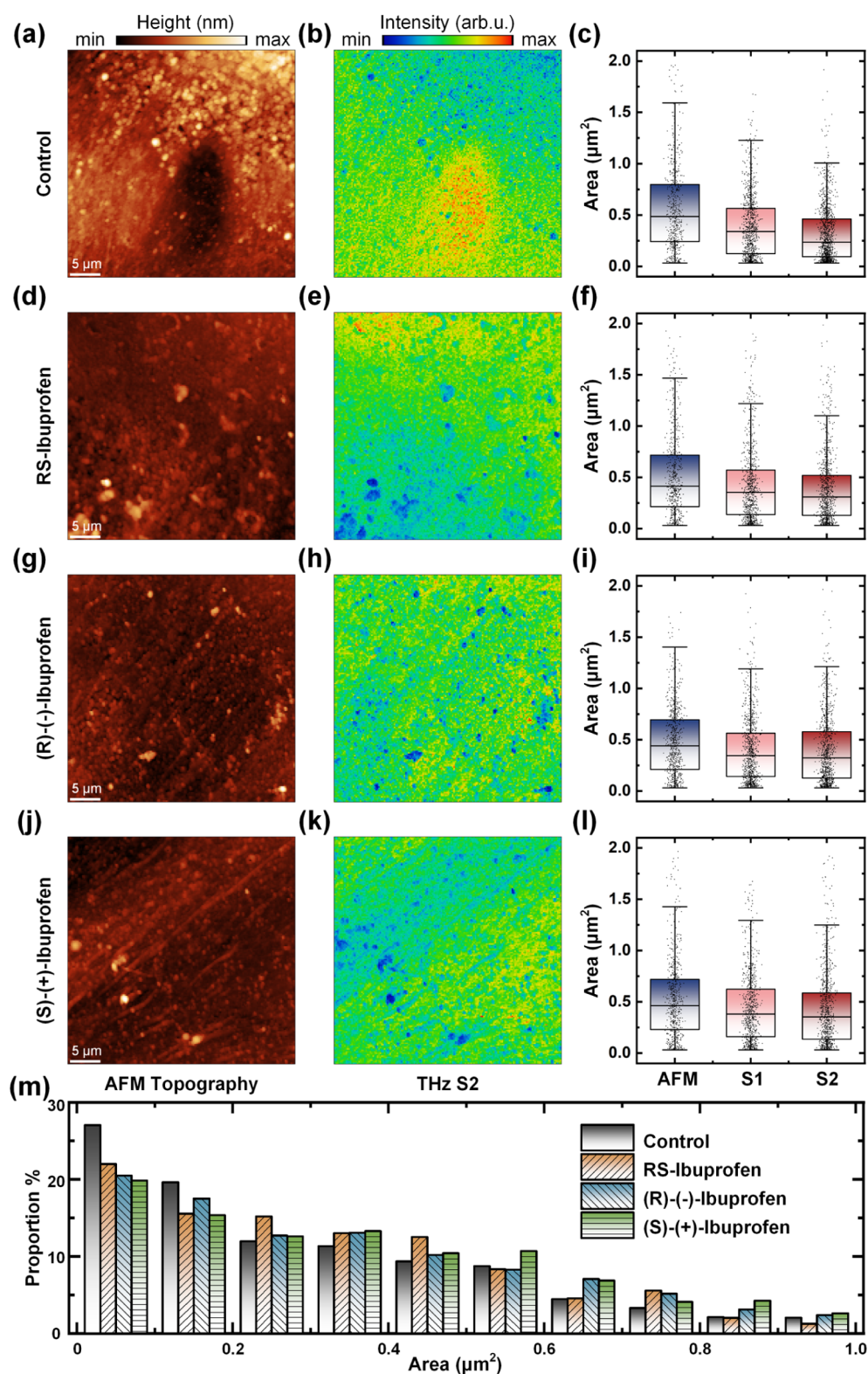


Figure 3. Nanoscale characterization and quantitative analysis of PEVs. (a, b), (d, e), (g, h), and (j, k) Comparative AFM topography (left panels) and corresponding THz near-field S2 amplitude maps (right panels) of the membrane region in control cell, RS-ibuprofen-treated cell, (R)-(-)-ibuprofen-treated cell, and (S)-(+)-ibuprofen-treated cell. (c, f, i, l) Quantitative analysis of PEV area distribution derived from three independent measurements: AFM topography (a, d, g, j) and THz S1 amplitude (Figure S3a–d) and THz S2 amplitude (b, e, h, k). Box plot elements represent the interquartile range (IQR, box body), the median value (central line), and $1.5 \times \text{IQR}$ whisker ranges. (m) Comparative histogram of submicron PEVs ($0\text{--}1.0 \mu\text{m}^2$) quantified from the THz S2 amplitude images across all experimental groups. Scale bars: 5 μm (all panels).

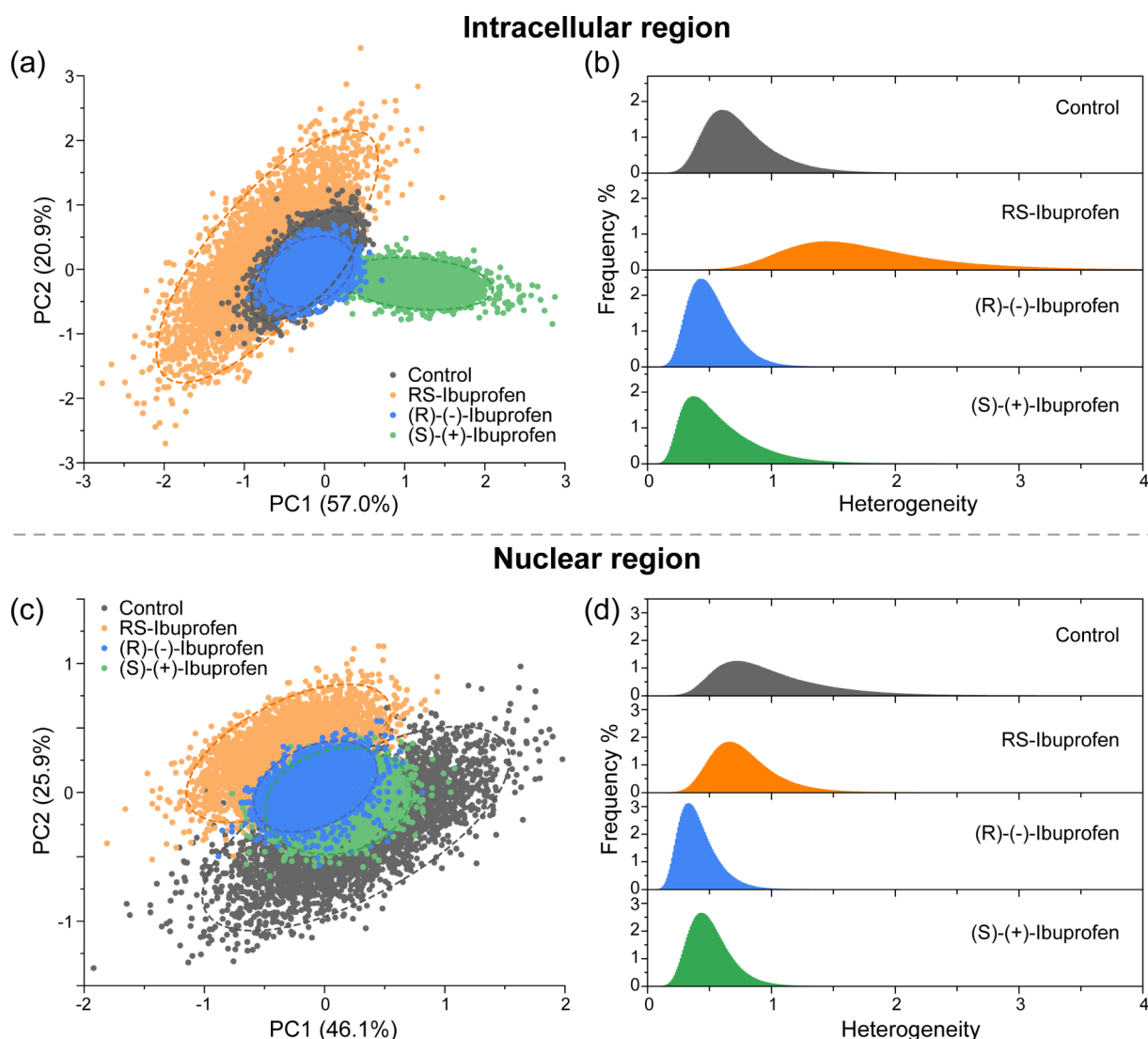


Figure 4. Multivariate analysis of THz absorption spectra for cells treated with chiral drugs. (a) Principal component analysis (PCA) and (b) the heterogeneity analysis on the intracellular region. (c, d) PCA and the heterogeneity analysis on the nuclear region.

Significance Testing. For near-field absorption spectra from the nuclear regions of control and chiral drug-treated cells, single-frequency absorption images were constructed and difference images were computed. GraphPad Prism software was used to perform a normality test and a homogeneity of variance test on the difference image data for the four cell groups. One-way ANOVA was performed to determine whether significant differences existed among the means of the spectral data of the different drug treatment groups, followed by Tukey's correction for multiple comparisons. Bar charts and error bars were created to show the mean values and standard errors for each group in order to visually compare the spectral data from the different treatment conditions.

K-Means Clustering Analysis. To further analyze the heterogeneity of the nuclear regions of control and chiral drug-treated cells, AFM topographical data and THz near-field absorption spectral intensity at the same location were constructed into a two-dimensional feature vector [height, absorption]. To avoid deviations caused by differences in feature scales, all of the features were normalized. K-means clustering analysis was performed in MATLAB, and the number of clusters was set to 4 (to effectively reflect the

differences between regions). The clustering outcomes were visualized using MATLAB software to show the data points in different categories, each with pseudocolor labeling. By calculating the proportion of each type of data point, we reveal the compositional differences in the cell nuclear region under different chiral drug treatments.

RESULTS AND DISCUSSION

THz Near-Field Images of Single Stem Cells. THz near-field images of representative stem cells treated with three chiral ibuprofen variants are presented in Figure 2. Figure 2a displays the AFM topographical image and corresponding THz near-field amplitude maps at first (S1), second (S2), and third (S3) harmonic demodulation frequencies for a single intact cell treated with racemic RS-ibuprofen. Figure 2b,c shows analogous results for cells treated with the enantiomers (R)-(-)-ibuprofen and (S)-(+)-ibuprofen, respectively. In the AFM topography, high-elevation regions (white contrast) correspond to the cell nucleus, whereas low-intensity zones (red contrast) in the THz amplitude maps align with nuclear localization. Notably, nuclei in all treatment groups were peripherally positioned, with the (S)-(+)-ibuprofen group

exhibiting nuclear displacement toward the narrow tail region of the cell.

AFM topography revealed distinct oval-shaped nuclear boundaries in the representative control cell,¹² RS-ibuprofen-treated cell, and (R)-(-)-ibuprofen-treated cell, with measured long/short-axis dimensions of 24.4 μm /14.0 μm , 19.5 μm /15.8 μm , and 32.1 μm /18.0 μm , respectively. THz near-field S1–S3 amplitude maps mirrored these nuclear contours. However, the representative (S)-(+)-ibuprofen-treated cell exhibited indistinct nuclear boundaries, elevated nuclear height, and increased surface pseudopodia density (filamentous protrusions), suggesting chiral-specific alterations in membrane morphology.

High-density vesicular protrusion structures were observed on cell membranes via both AFM and THz imaging (Figure 3). Their morphological characteristics are similar to PEVs reported in the literature.¹⁵ AFM topography identified PEVs as elevated circular domains (white contrast), while THz amplitude maps depicted them as low-intensity circular features (blue contrast). Four subregions were selected in each cell for the PEV analysis. Figures 3 and S3 show the AFM and THz near-field images of region 1 of each representative cell, while Figures S4–S7 present the corresponding images of the remaining three regions of these four cells. Based on lateral contour analysis, we obtained the PEV area. Statistical analysis focused on PEVs with diameters <800 nm (i.e., areas smaller than 2.01 μm^2), as larger vesicles were infrequent.¹⁶ The statistical results of the PEV area in region 1 of each representative cell are shown in Figure 3c,f,i,l. The results showed that the median PEV area from AFM topography (0.485 μm^2 control, 0.415–0.463 μm^2 drug-treated) consistently exceeded THz-derived values (0.236–0.381 μm^2), which is related to the fact that the THz near-field amplitude image provides a high lateral contrast for the differences in sample composition.¹²

THz S2 amplitude maps (Figure 3b,e,h,k) further demonstrated that >92% of PEVs had areas <1.0 μm^2 . Comparative analysis (Figure 3m) showed that chiral drug treatment significantly increased the median PEV area relative to control: RS-ibuprofen (+24.6%), (R)-(-)-ibuprofen (+25.4%), and (S)-(+)-ibuprofen (+39.9%). The proportion of the PEV area in the 0.3–1.0 μm^2 range increased from 41.4% (control) to 47.3–52.2% (drug-treated), indicating enantiomer-dependent vesicle enlargement. Specifically, (S)-(+)-ibuprofen treatment yielded the largest PEVs (28.5% in the 0.5–1.0 μm^2 range vs 21.7% for the RS-ibuprofen and 26.0% for the (R)-(-)-ibuprofen), consistent with its higher bioactivity.^{17,18} Furthermore, the median PEV area (range 0–1.0 μm^2) calculated from regions 2–4 of each representative cell is summarized in Table S1, further supporting the above trend.

The observed PEV size modulation aligns with prior reports of drug-induced extracellular vesicle remodeling. For instance, ketamine exposure increases microvesicle diameters by ~2-fold (217.5 to 400.0 nm),¹⁹ while membrane-active compounds like ibuprofen may alter lipid bilayer dynamics,²⁰ secretory pathway,²¹ and miRNA-regulated vesicle biogenesis.^{22,23} We propose that (S)-(+)-ibuprofen's potent cyclooxygenase (COX) inhibition, via suppression of prostaglandin synthesis,²⁴ contrasts with the weaker activity of its (R)-enantiomer, which undergoes metabolic inversion to the active (S)-(+)-ibuprofen.¹⁸ This stereospecific interaction likely perturbs membrane fluidity, receptor signaling, and vesicle trafficking mechanisms, driving differential PEV secretion.

These findings underscore the necessity to evaluate chiral drug–membrane interactions comprehensively, particularly for mesenchymal stem cell applications. Future investigations should delineate molecular targets (e.g., COX isoforms and lipid rafts) and downstream pathways governing PEV biogenesis, advancing therapeutic strategies leveraging chiral specificity.

Analysis of Single-Cell Heterogeneity Under Chiral Drug Treatment. To elucidate the mechanisms underlying chiral drug treatment, we focused on two key regions: the intracellular region (comprising the cytoplasm and membrane) and the nuclear region (including the nucleus and its surrounding areas), as shown in Figure S8. Spectral data from these regions were collected for the four experimental groups (Figure S9 presents spectra from four test points of each representative cell as an example) and analyzed using PCA. The PCA results are presented in Figure 4a,c, with detailed metrics provided in Table S2. These metrics include the mean, standard deviation, and confidence ellipse area for each group in the PC1 and PC2 directions, as well as the intergroup centroid distance, which quantifies the spectral differences between groups.

In the intracellular region, PC1 and PC2 collectively accounted for 77.9% of the total variation, indicating that these components effectively captured the main changes in the spectral features. The control group exhibited a small confidence ellipse area (1.3916), reflecting minimal spectral variation among test points and suggesting a relatively homogeneous composition in the membrane region. In contrast, the RS-ibuprofen-treated group displayed the largest standard deviations in PC1 and PC2 and the largest confidence ellipse area (5.5586). These results indicate significant spectral heterogeneity in the intracellular region following RS-ibuprofen treatment, likely due to substantial changes in the chemical composition induced by the drug.

The spectral characteristics of the (R)-(-)-ibuprofen group closely resembled those of the control group, with comparable mean and standard deviation values for PC1 and PC2. The small intergroup centroid distance (0.1071) further confirmed minimal compositional changes in the membrane region. For the (S)-(+)-ibuprofen group, although the confidence ellipse area was similar to that of the control group, the mean PC1 value shifted significantly to 1.1607 (compared with -0.2339 for the control group, -0.7482 for the RS-ibuprofen group, and -0.1786 for the (R)-(-)-ibuprofen group). Additionally, the centroid distance between the (S)-(+)-ibuprofen group and the control group was the largest (1.4148), indicating significant spectral differences in the intracellular region. These changes, however, were consistent across measurement points, suggesting uniform compositional alterations induced by (S)-(+)-ibuprofen.

To further quantify cellular heterogeneity under chiral drug treatment, we performed heterogeneity analysis based on Euclidean distances (see the Materials and Methods section). Figure 4b,d illustrates the inner-group heterogeneity for each group. The RS-ibuprofen group exhibited the lowest peak position (0.7959) and the highest peak intensity (1.45), consistent with the PCA results and indicating pronounced spectral heterogeneity in the intracellular region. In contrast, the control, (R)-(-)-ibuprofen, and (S)-(+)-ibuprofen groups showed higher peak positions (1.7529, 2.4464, and 1.8786, respectively) and low heterogeneity values (0.60, 0.43, and 0.37, respectively). The median heterogeneity values for these

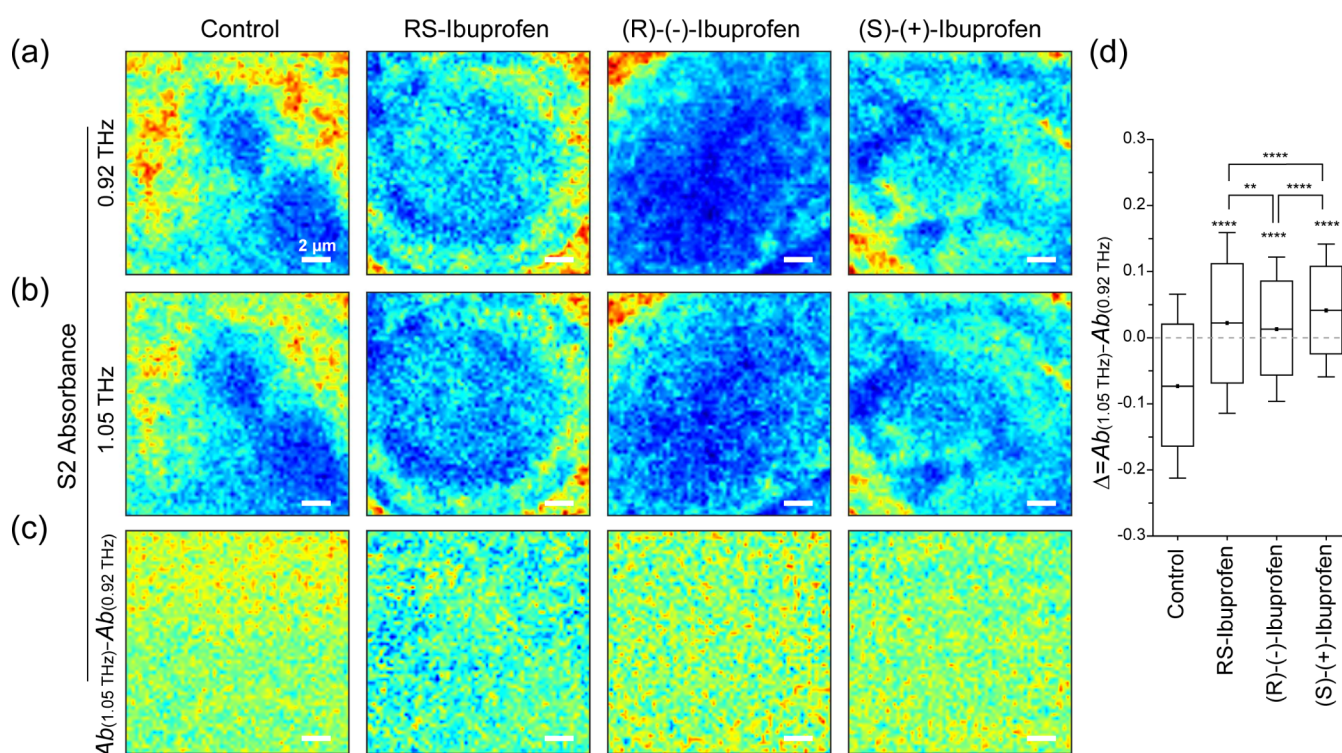


Figure 5. THz near-field absorption imaging of nuclear regions at specific frequencies across the four experimental groups. (a, b) THz near-field S2 absorption images of nuclear regions at 0.92 and 1.05 THz for the four groups. (c) Difference images derived by subtracting absorption at 0.92 THz from absorption at 1.05 THz, highlighting spectral variations in the nuclear region. (d) Statistical analysis of absorption intensity differences between 1.05 and 0.92 THz for each test point within the nuclear regions. Significance levels are denoted as follows: ** $p < 0.01$, **** $p < 0.0001$.

groups were 0.70, 0.50, and 0.51, respectively. The similarity in peak positions and median values between the (R)-(-)-ibuprofen and (S)-(+)-ibuprofen groups and the control group suggests that these drugs induced comparable trends in spectral changes, with minimal structural differences occurring within the intracellular region.

In the nuclear region, PC1 and PC2 explained 71.8% of the total variation. The control group exhibited a confidence ellipse area of 2.4367, indicating substantial spectral variability and a diverse nuclear composition. In contrast, all chiral drug-treated groups showed smaller confidence ellipse areas, reflecting reduced spectral variability and a tendency toward nuclear homogenization. Specifically, the (R)-(-)-ibuprofen group displayed the smallest confidence ellipse area (0.5340) and the highest peak intensity (3.1072) in inner-group heterogeneity, indicating minimal heterogeneity and a highly uniform nuclear composition.

The RS-ibuprofen group exhibited a larger confidence ellipse area (1.1604) and a lower peak intensity (1.8232) compared with the other drug-treated groups, suggesting greater spectral variation and a less pronounced trend toward homogenization. This group also had the largest intergroup centroid distance from the control group (0.8082), highlighting significant compositional differences. In contrast, the (S)-(+)-ibuprofen group showed the smallest centroid distance (0.2581), indicating that its nuclear spectral properties were most similar to those of the control group despite a trend toward reduced nuclear diversity.

In summary, PCA and heterogeneity analyses of THz near-field spectral data revealed distinct effects of chiral drugs on cell heterogeneity. RS-ibuprofen significantly increased the

intracellular compositional variability while reducing nuclear heterogeneity. Both (R)-(-)-ibuprofen and (S)-(+)-ibuprofen maintained membrane uniformity but differentially altered nuclear composition. (R)-(-)-ibuprofen had a minor impact on intracellular composition, whereas (S)-(+)-ibuprofen induced notable membrane differences compared with the control group. Interestingly, their effects on nuclear regions exhibited an opposite trend, suggesting a distinct mechanism of action for these enantiomers. These findings provide valuable insights into the cellular response to chiral drugs and their potential biological mechanisms.

Analysis of Nuclear Composition Under Chiral Drug Treatment. The cellular nucleus, as the center hub of genetic information, not only regulates gene expression but also directly influences cellular functions and the synthesis of secretory substances.¹⁹ Therefore, analyzing compositional changes within the nucleus is crucial for elucidating how chiral drugs modulate cellular function and secretion. In this section, we focus on a quantitative analysis of the heterogeneity of nuclear responses to chiral drugs. To visualize these changes, we utilized THz near-field absorption images at specific frequencies.

As discussed in the previous section, cells exhibited distinct biological reactions to different chiral drugs. Specifically, (R)-(-)-ibuprofen had a minimal impact on cell membrane heterogeneity but significantly altered nuclear heterogeneity. In contrast, RS-ibuprofen increased cell membrane heterogeneity, while it reduced nucleus heterogeneity. Additionally, chiral drugs exhibited unique spatial distributions within cells during their action.²⁵ These results suggest that chiral drugs may induce changes in the components of the nuclear region, and it

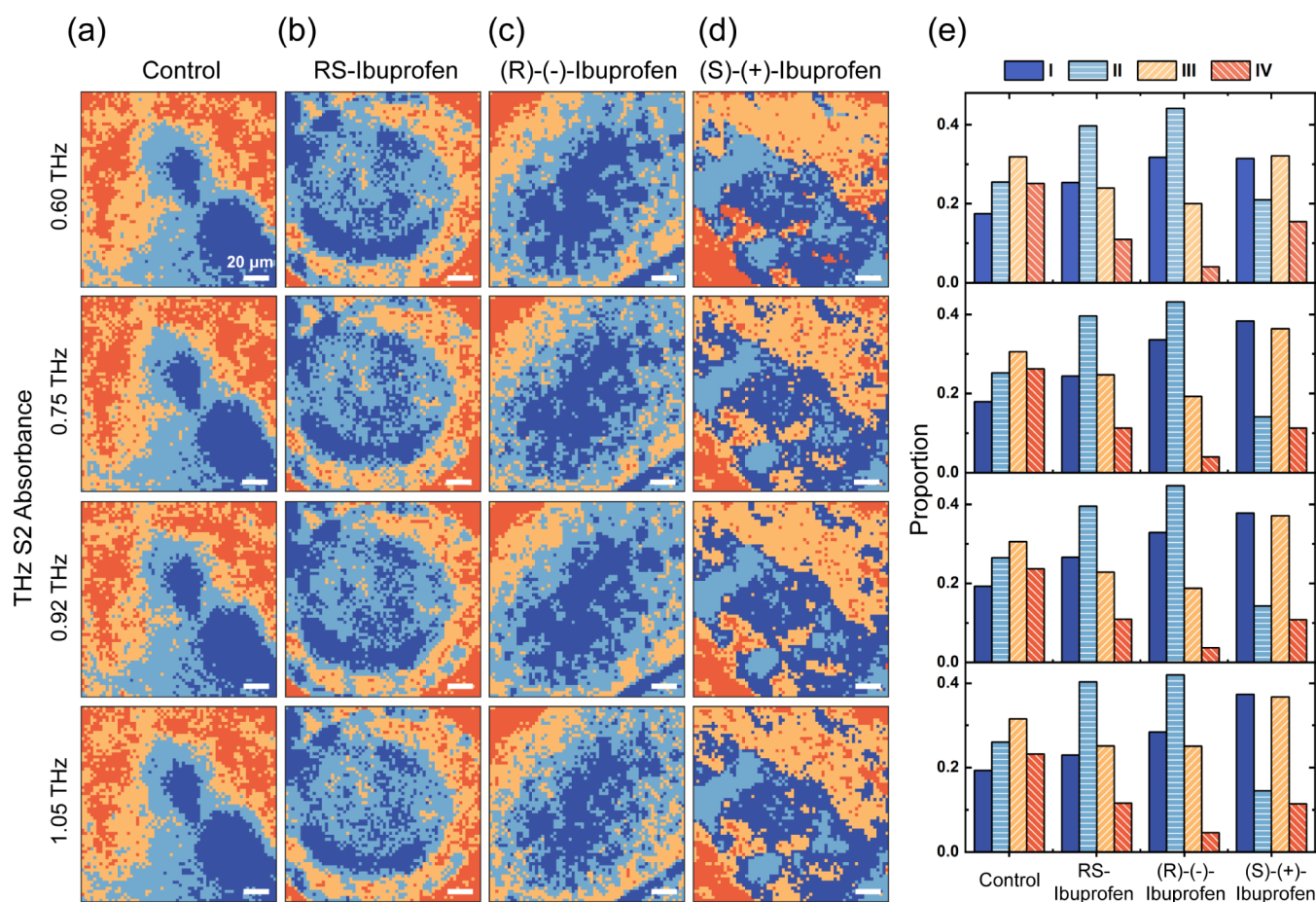


Figure 6. Analysis of nuclear components. (a)–(d) Cluster analysis results for four experimental groups, displaying the spatial distribution of nuclear components. Rows 1–4 represent the clustering patterns derived from single-frequency absorption images at 0.60, 0.75, 0.92, and 1.05 THz, respectively. (e) Quantitative statistical analysis of cluster distributions across the four frequency points, as derived from the clustering results in (a)–(d).

is necessary to further conduct quantitative visualization analyses based on THz absorption characteristics. Racemic RS-ibuprofen exhibits an absorption peak at 1.05 THz, while its enantiomers, (R)-(–)-ibuprofen and (S)-(+)-ibuprofen, show absorption peaks at 0.92 THz.²⁶ However, these characteristic peaks were not significant in the absorption spectra (Figure S9). We tried to construct THz near-field single-frequency absorption images of nuclear regions at 0.92 and 1.05 THz, as shown in Figure 5a,b. Due to the low residual drug content in single cells, the differences in single-frequency images were subtle. To enhance spectral contrast, we computed difference images between 0.92 and 1.05 THz, as presented in Figure 5c,d.

In Figure 5c, red areas indicate higher absorption at 1.05 THz compared with 0.92 THz. For the control group, approximately 37.4% of the image areas exhibited differences smaller than -0.1 , while only 3.2% showed differences greater than 0.1 . In contrast, for cells treated with chiral drugs, the proportions with differences smaller than -0.1 were 8.9% (RS-ibuprofen), 6.4% [(R)-(–)-ibuprofen], and 2.4% [(S)-(+)-ibuprofen]. The proportions with a difference greater than 0.1 were 18.5% for the RS-ibuprofen group, 11.5% for the (R)-(–)-ibuprofen group, and 20.0% for the (S)-(+)-ibuprofen group. For the control cell, the regions with a negative value accounted for 78.7%, indicating that the THz absorption of the nucleus was greater at 0.92 THz than at 1.05 THz. In contrast,

for the drug-treated cells, the regions with positive values accounted for 60.0% for the RS-ibuprofen group, 57.5% for the (R)-(–)-ibuprofen group, and 72.0% for the (S)-(+)-ibuprofen group. The higher proportion of positive values suggested that the THz absorption of the nucleus was lower at 0.92 THz than at 1.05 THz. These results indicate that the absorption characteristics of the nuclear regions treated with chiral drugs have changed, and each treatment group shows different distribution trends of absorption differences. Although the current data are not yet sufficient to clearly attribute to specific molecular mechanisms or changes in nuclear components, this spectral difference suggests potential drug effects, which need to be further clarified through more systematic spectral analysis and biochemical validation.

To further quantify these effects, we performed statistical analysis on the different images (Figure 5d). The mean values and standard deviations were as follows: control group (-0.0733 ± 0.1392), RS-ibuprofen group (0.0223 ± 0.1366), (R)-(–)-ibuprofen group (0.0128 ± 0.1092), and (S)-(+)-ibuprofen group (0.0412 ± 0.1005). The RS-ibuprofen-treated group exhibited the highest mean and standard deviation, indicating the most pronounced changes in nuclear composition. This resulted in greater differences in single-frequency absorption images compared to the control group. Furthermore, as described in the Materials and Methods section, significant absorption differences were

observed among the four groups, confirming that single-frequency difference images can effectively distinguish drug-treated cells from control cell. These results demonstrate that chiral drug treatments induce measurable changes in nuclear composition, providing a foundation for studying cellular responses to drug treatments, mapping the spatial distribution of drugs within cells, and exploring the functional implications of nuclear composition changes. Thus, the analysis advances our understanding of how chiral drugs influence cellular behavior at the molecular level and offers new insights into their mechanisms of action.

To quantitatively assess the heterogeneity of cellular responses to chiral drugs in the nuclear region, we further conducted a detailed analysis using K-means clustering analysis. Applying the methodology described in the [Materials and Methods](#) section, we generated four-class clustering maps (designated as I, II, III, and IV) at characteristic frequencies of 0.60, 0.75, 0.92, and 1.05 THz, as presented in [Figure 6a–d](#). For comparative analysis, we quantified the proportional distribution of each cluster across these frequencies for different treatment groups, with the statistical results in [Figure 6e](#).

In the representative control cell ([Figure 6a](#)), cluster I (deep blue) exhibited an elliptical distribution pattern, with proportions of 17.5%, 17.9%, 19.3%, and 19.3% at the respective frequencies (0.60, 0.75, 0.92, and 1.05 THz, respectively), yielding an average of $18.5 \pm 0.9\%$. The corresponding averages for clusters II, III, and IV were $25.8 \pm 0.5\%$, $31.1 \pm 0.7\%$, and $24.6 \pm 1.4\%$, respectively. The substantial variation observed in clusters I and IV suggests a frequency-dependent response, potentially reflecting the presence of multiple THz absorption peaks from different molecular components. The minimal fluctuation in cluster II indicates relatively constant THz absorption across the measured spectrum, while the pronounced variation in cluster IV implies the existence of characteristic absorption features within this frequency range.

Analysis of the RS-ibuprofen-treated group ([Figure 6b](#)) revealed distinct clustering patterns, with average proportions of $24.9 \pm 1.6\%$, $39.8 \pm 0.4\%$, $24.2 \pm 1.0\%$, and $11.2 \pm 0.3\%$ for clusters I–IV, respectively. The spectral characteristics suggest that clusters I and III contain components with significant absorption peaks in this range, while clusters II and IV show relatively flat spectral responses. Similarly, in the (R)-(–)-ibuprofen-treated cell, clusters I–III exhibited characteristic absorption peaks, contrasting with the nonabsorbing nature of cluster IV. Notably, all clusters in the (S)-(+)-ibuprofen-treated cell demonstrated distinct absorption peaks, indicating comprehensive molecular modification.

These clustering patterns provide valuable insights into the drug-induced alternations in nuclear composition, which appear to correlate with specific molecular regulatory mechanisms. Although the specific molecular composition of each cluster cannot be directly determined from the current data, the changes in the spectral response patterns among treatment groups suggest that chiral drug treatments may influence the compositional or structural characteristics of the nuclear region. The observed changes may be associated with (S)-(+)-ibuprofen's dual effects on β -catenin inhibition and NF- κ B-p65 nuclear translocation.²⁷ However, further biochemical validation is needed to confirm these associations. Through these clustering analyses, we have not only mapped the dynamic reorganization of nuclear components following

drug treatment but also established a connection between these structural changes and cellular adaptive responses. Although the analysis is limited by the influence of topography and the challenge of selecting fully representative nuclear regions, this approach offers a novel framework for understanding single-cell pharmacological responses and contributes to the development of precision medicine strategies by elucidating the nanoscale mechanism of drug action.

CONCLUSION

This study, leveraging the advanced THz s-SNOM platform, provides a comprehensive investigation into the effects of chiral drugs at the single-cell level, revealing previously uncharacterized heterogeneity in cellular responses to these compounds. The THz s-SNOM technique offers distinct advantages over traditional detection methods, providing nanoscale spatial resolution while maintaining label-free and noninvasive measurement capabilities.

Our findings demonstrated significant alterations in PEVs following chiral drug treatment with (S)-(+)-ibuprofen, showing particularly pronounced effects on PEV secretion and area expansion. Through PCA and spectral heterogeneity analysis, we identified distinct variations in the cell membrane and nuclear regions across different chiral drug treatments. Notably, RS-ibuprofen exhibited the most substantial impact on both the cell morphology and biochemical composition.

We further quantified the heterogeneity of cellular responses to chiral drugs through single-frequency absorption imaging and cluster analysis. These approaches enabled clear discrimination between drug-treated and control cells, revealing significant drug-induced modifications in the nuclear composition. The observed changes in nuclear components provide valuable insights into the structural and functional alterations induced by chiral drugs at the subcellular level. This work established THz s-SNOM as a powerful tool for nanoscale imaging, offering novel perspectives for investigating drug–cell interactions and intracellular evolutionary pathways. These methodologies and findings presented here contribute significantly to the technical and theoretical foundation for chiral drug development and screening, potentially advancing precision medicine approaches in pharmaceutical research.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.5c01999>.

Median PEV area from regions 1–4 of each representative cell (Table S1); PCA results of THz absorption spectra (Table S2); optical microscope images of three drug-treated cells (Figure S1); AFM topographical images of three drug-treated cells (Figure S2); THz near-field S1 amplitude maps of the membrane region in four groups (Figure S3); AFM topography and THz near-field amplitude maps of regions 2–4 from a representative control cell (Figure S4); AFM topography and THz near-field amplitude maps of regions 2–4 from a representative RS-ibuprofen-treated cell (Figure S5); AFM topography and THz near-field amplitude maps of regions 2–4 from a representative (R)-(–)-ibuprofen-treated cell (Figure S6); AFM topography and THz near-field amplitude maps of regions 2–4 from a representative (S)-

(+)-ibuprofen-treated cell (Figure S7); AFM topography and corresponding THz near-field S2 amplitude maps of the intracellular region and nuclear region in four groups (Figure S8); THz near-field absorption spectra of representative cells (Figure S9) (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

THz	terahertz
s-SNOM	scattering-type scanning near-field optical microscopy
AFM	atomic force microscopy
PEVs	plasma membrane-derived extracellular vesicles
S1	first harmonic demodulation
S2	second harmonic demodulation
S3	third harmonic demodulation
PCA	principal component analysis

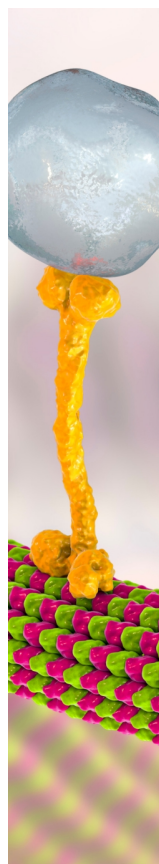
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