Supplementary Information

Large-scale 3D optical mapping and quantitative analysis of nanoparticle distribution in tumor vascular microenvironment

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Figure S1. Characterization of MSNs. (a) Hydrodynamic volume of unmodified MSN, amine-functionalized MSN (MSN-NH$_2$), and FITC-conjugated MSN (MSN-FITC). (b) Zeta potential of unmodified MSN, MSN-NH$_2$, and MSN-FITC. (c) Calibration curve of FITC-conjugated MSNs ($n = 3$ for each). Dotted line indicates the fitting curve obtained by linear regression. Data are mean ± s.e.m.
Figure S2. Optical clearing of NP-containing organs. FITC-conjugated MSN$_{200}$ was systemically injected, and after 24 hrs, tissues were harvested, cut into 1 mm-thick sections, and processed by the OMNIA procedure. Representative images of tumor, liver, kidney, and spleen showing distinct distribution patterns of NPs. Scale bars, 50 μm (top panels), 20 μm (bottom panels).
Figure S3. The OMNIA process does not compromise fluorescence and the detectability of NPs.

(a) Representative bright-field images of a liver sample without or with clearing. Grids, 1 mm.

(b) Representative confocal images of the liver surface, showing comparable fluorescence intensity for both NPs (green, FITC-conjugated MSN₂₀₀) and blood vessels (red, stained with DyLight 649-conjugated tomato lectin) without or with clearing. (c) Mean intensities of FITC signals measured from NP spots from tissues with or without clearing process were not different ($p = 0.4557$, $n = 7$ samples, Mann-Whitney $U$ test). (d) Mean intensities of DyLight 649 signals of stained blood vessels were also similar without or with clearing ($p = 0.6183$, $n = 7$ samples, Mann-Whitney $U$ test). (e) Volume imaging is possible only in the cleared, but not
in the uncleared samples. Note comparable signals of blood vessels and NPs at both the surface and core of the sample. Data are mean ± s.e.m. Scale bars, 20 μm (b), 100 μm (e, volume), 20 μm (e, plane images).
**Figure S4.** Comparable detection of blood vessels and NPs throughout the depth of the liver. (a) Representative 3D image of liver. (b) Probability distribution of blood vessel density and NP concentration over z-depth of the liver tissue. Blood vessel density ($p = 0.5254$, Kruskal-Wallis test) and NP concentration ($p = 0.0925$, Kruskal-Wallis test) were not significantly different throughout the depth. $n = 5$ for each. Data are mean ± s.e.m. Scale bars, 200 μm (a, left), 30 μm (a, right).
**Figure S5.** Transcardial perfusion does not affect NP intensity and distribution. FITC-conjugated MSN$_{200}$ was systemically injected, and after 24 hrs, mice were perfused or not, and the liver was harvested. (a) Representative confocal images of the surface of the liver tissue without or with transcardial perfusion showing comparable NP intensity and distribution. (b) Mean intensities of FITC signals measured from NP spots were similar without or with perfusion ($p = 0.8413$, $n = 5$ samples, Mann-Whitney $U$ test). (c) % extravasated NPs were also similar without or with perfusion ($p = 0.8413$, $n = 5$ samples, Mann-Whitney $U$ test). Data are mean ± s.e.m. Scale bars, 20 μm.
Figure S6. Comparisons of optical images obtained with a low-numerical aperture (NA), long-working distance (WD) objective lens (10×, 0.5 NA, 3.7 mm WD) with high-NA, short-WD lens (40×, 1.2 NA, 0.28 mm WD). (a) Representative confocal images of fluorescently labeled NPs acquired with 10× lens (green) and 40× lens (red), showing the overlap between the two images. (b) Gaussian-fitted intensity profiles of white lines in (a), demonstrating that high-NA objective lens (40×) discriminated two chunks of NP fluorescence more sharply. Thus, high-NA lens has a better resolution power, and would better resolve individual NPs or their clusters. (c) The numbers of the chunks of NP fluorescence were not significantly different between the images acquired with low-NA (10×) or high-NA (40×) objective lenses (p > 0.9999, n = 5 samples, Mann-Whitney U test). Here, detectability was defined as the ratio of the number of fluorescent chunks detected from the images acquired with the low-NA (10×) or high-NA (40×) objective lenses, to the number of those detected from the separate set of images obtained with high-NA (40×) objective lenses in a different experimental session. We speculate that most NPs existed in the form of clusters in our experiments, such that both the low- or high-NA objective lens could detect the NP fluorescence to a similar extent. Alternatively, it is possible that the fluorescence signal from individual NPs were sufficiently bright to allow
for faithful detection even with the low-NA objective lens in our experimental conditions.

Data are mean ± s.e.m. Scale bars, 2 μm.
Figure S7. Size distribution of fluoresently detected NPs in tumor tissue. (a) Representative confocal fluorescence image of NPs without (left) or with 3D-reconstructed NP spots (red spheres) (right), showing faithful representation of fluorescent spots by NP spots. (b) Distribution of full width at half maximum (FWHM) of randomly chosen 200 fluorescent spots from tumor tissues ($n = 3$ samples). The smallest observable size was determined by the optical resolution limit of the objective lens (~400 nm). The mean value of FWHM was $766 \pm 317$ nm (mean ± s.d.). Scale bars, 5 μm.
Figure S8. 3D volume image allows more precise measurement of penetration distance from the blood vessels. (a) Example color map showing the distance from the blood vessel surface, for 10 and 300 μm-thick optical sections from the same sample, demonstrating an overestimation of the distance from blood vessel surface for thinner sections. Arrows and arrowheads denote noticeable differences. (b) Mean penetration distance of extravasated NPs were significantly shorter in 300 μm-thick optical sections than in 10 μm sections ($p = 5.76 \times 10^{-9}$, $n = 496$ NPs for each, unpaired $t$-test). Right, examples of penetration distance measurement. Measurement from 10 μm-thick optical sections overestimates the penetration distance of extravasated NPs, since the closest blood vessel may not remain in the same optical sections. (c) Cumulative probability distribution of penetration distance for extravasated NPs ($p = 0.0364$ for interaction, $n = 7$ for each, two-way repeated-measures ANOVA). Data are mean ± s.e.m. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Scale bars, 10 μm (a), 5 μm (b).
Figure S9. Time-course analysis of NP accumulation and retention in liver. (a) Representative optical section and 3D reconstruction images of liver tissues at 1, 24, and 72 hrs after systemic injection of NPs (green, FITC-conjugated MSN$_{200}$). (b) Extravasated NP concentration peaked at 24 hrs and considerably reduced at 72 hrs ($p = 0.0006$). (c) % extravasated NPs also peaked at 24 hrs after injection ($p = 0.3180$). (d) Penetration distance was not significantly change with different timepoints ($p = 0.0097$). $n = 3$ for 1 hr, $n = 10$ for 24 hrs, $n = 4$ for 72 hrs. **$p < 0.01$. Scale bars, 20 μm.
Figure S10. Cancer cell co-visualization with blood vessel and NPs. Cancer cells were visualized by transplanting GFP-transfected HeLa cells (cyan pseudocolor). AlexaFluor647-conjugated NPs were delivered (green pseudocolor). a) Tumor 3D image co-visualizing blood vessel (red), cancer cell, and NP. Necrotic center shows lack of viable cancer cells, and also blood vessel penetration. b) The number of cancer cell were counted. c) Cumulative frequency of cancer cell by the distance from blood vessel. Scale bar, 200 μm (a).