Quantitative imaging of intracellular nanoparticle exposure enables prediction of nanotherapeutic efficacy

Nanoparticle internalisation is crucial for the precise delivery of drug/genes to its intracellular targets. Conventional quantification strategies can provide the overall profiling of nanoparticle biodistribution, but fail to unambiguously differentiate the intracellularly bioavailable particles from those in tumour intravascular and extracellular microenvironment. Herein, we develop a binary ratiometric nanoreporter (BiRN) that can specifically convert subtle pH variations involved in the endocytic events into digitised signal output, enabling the accurately quantifying of cellular internalisation without introducing extracellular contributions. Using BiRN technology, we find only 10.7–28.2% of accumulated nanoparticles are internalised into intracellular compartments with high heterogeneity within and between different tumour types. We demonstrate the therapeutic responses of nanomedicines are successfully predicted based on intracellular nanoparticle exposure rather than the overall accumulation in tumour mass. This nonlinear optical nanotechnology offers a valuable imaging tool to evaluate the tumour targeting of new nanomedicines and stratify patients for personalised cancer therapy.
Nanotherapeutics have made significant contributions in oncology through precise delivery of drugs into specific tissues with mitigated side-effects. Over ten nanomedicines have been approved for cancer treatments, including PEGylated liposomal doxorubicin (Doxil/Caelyx), paclitaxel-albumin nanoparticle (Abraxane), etc. However, cancer patients presented heterogeneous therapeutic responses to nanomedicines in clinical trials due to the extreme variability of the complex tumour microenvironment. Efforts have been made to identify cancer patients with high EPR effect to achieve remarkable therapeutic response. Nevertheless, an important truth that cannot be ignored is nanomedicines with high tumour accumulation sometimes fail to achieve enhanced therapeutic response due to the inadequate target exposure (e.g. Doxil versus free doxorubicin). It is increasingly clear that drug exposure at the intracellular targets rather than the overall level in the tumour tissues acts as one of the most important factors to achieve the optimal therapeutic response. Thus, quantifying the intracellular bioavailability of nanomedicines, including internalised amount and percentage in solid tumours would be a suitable indicator for the prediction of drug access to intracellular targets and therapeutic efficacy.

Conventional methods based on fluorescence spectroscopic, mass spectrometric and radioactive detections have been extensively used to quantify the accumulation of nanomedicines in solid tumours. However, these methodologies fail to offer the information on the intracellular delivery efficiency of accumulated nanoparticles in tumour mass due to the incapability of differentiating the internalised nanoparticles from those in tumour intravascular and extracellular spaces. Several strategies such as chemical etching, fluorescence quenching and bioluminescence reaction have been extensively leveraged to exclude the extracellularly distributed nanoparticles from endocytosis analysis, and provide two-dimensional (2D) information at cellular or ex vivo level. Despite the successful quantification of the nanoparticle internalisation in vitro, achieving real-time quantification of nanomedicines internalisation kinetics in living subjects remains a significant challenge.

Herein, we present a binary ratiometric nanoreporter (BiRN) that can unambiguously differentiate intracellularly and extracellularly located nanoparticles, permitting quantitative imaging of nanoparticle internalisation in vivo (Fig. 1a, b). We chose a well-recognised endocytosis signal, acidic early endosomal pH (pH_e ~ 6.0) to report nanoparticle internalisation events due to the fast formation of early endosome after endocytosis (~5 min). Our BiRN is a two-modular nanoreporter system with a transition pH (pH_t) at 6.323, and each module is fluorescently encoded with a unique near-infra-red fluorophore. The ‘OFF-ON’ module keeps ‘OFF’ in tumour bloodstream and extracellular space (pH_e ~ 6.6–6.9), and immediately switches ‘ON’ upon a sudden pH drop in early endosome after endocytosis. The ‘always-ON’ module acts as an internal standard for ratiometric quantitation of nanoparticle internalisation. After binary ratiometric processing, the nanoparticles in tumour microvasculature and extracellular space were removed, allowing the real-time measurement of the cellular internalisation in living mice. Enabled by BiRN, we demonstrate the successful

![Fig. 1 Design and mechanism of BiRN for real-time quantitative imaging of cellular internalisation in vivo.](https://example.com/fig1.png)

**Fig. 1 Design and mechanism of BiRN for real-time quantitative imaging of cellular internalisation in vivo.** a The schematic of internalisation and activation of BiRN nanoparticles in living mice. BiRN comprises ‘OFF-ON’ and ‘always-ON’ fluorescence modules, serving as a binary pH threshold nanoreporter at pH 6.3. ‘OFF-ON’ module stays ‘OFF’ in tumour microcirculation and extracellular space (pH_e = 6.6–6.9), and immediately turns ‘ON’ in early endosome (pH ~ 6.0) after endocytosis. ‘Always-ON’ module keeps constant fluorescence intensity regardless of extra- and intracellular distribution. The ratiometric images (F_{OFF-ON}/F_{always-ON} O/A) remove all the extracellular signals and only internalised nanoparticles can be quantified (white punctate) after binary processing. b The ratiometric O/A signal of BiRN is a binary output (0 and 1) reporting the micro-distribution of nanoparticle in the extracellular (0) and intracellular (1) space. c Based on BiRN, we stratify cancer patients according to endocytosis amount and predict successfully the nanotherapeutic outcomes.
quantification of nanoparticle internalisation in various animal tumour models. Furthermore, the prediction of therapeutic outcomes of cancer nanomedicines, including paclitaxel (PTX)-conjugated nanoparticle, which has identically physicochemical properties to BiRN, as well as Doxil was accomplished by patient stratification based on intracellular nanoparticle exposure in tumour mass (Fig. 1c). These capabilities make the present technology particularly potent in the comprehensive understanding of the Nano–Bio interaction in the tumour microenvironment and personalised cancer nanomedicine.

Results

Design and characterisation of the BiRN. We synthesised the poly(ethylene glycol)-b-poly(2-(diisopropyl amino)ethyl methacrylate) (PEG-b-PDPA) polymer with a pH₃ at 6.3 via the atom transfer radical polymerisation method (Supplementary Fig. 1).25 Several dye-conjugated polymers were synthesised and divided into two groups, i.e. ‘always-ON’ modular polymer (F₀max/F₀min < 10-fold, F₀max and F₀min represent the maximal and minimal fluorescence intensities at unimer and micelle states, respectively.) and ‘OFF-ON’ modular polymer (F₀max/F₀min > 100-fold) (Supplementary Table 1 and Supplementary Figs. 2, 3). In an optimised BiRN, the dyes in the ‘OFF-ON’ and ‘always-ON’ modules should be FRET donor and acceptor, respectively (Fig. 2a). To generate the ‘always-ON’ signal, a low molar fraction of ‘always-ON’ modular polymer was used in nanoparticles to abolish homofRET-induced fluorescence quenching (Supplementary Fig. 4). We successfully constructed four pH-sensitive BiRN nanoparticles that meet the following criteria: the fluorescence signal of ‘always-ON’ module keeps constant (F₀max/F₀min = 0.9–1.1) at pH ranging from 5.0 to 7.4; the ‘OFF-ON’ module renders two-order magnitude signal amplification (F₀max/F₀min > 100-fold) when pH drops below the threshold of 6.3 (Supplementary Fig. 5); the pH-responsiveness of nanoparticle is very sharp (ΔpHₚHOFF < 0.25) (Supplementary Table 2). In the following experiments, BiRN consisted of PDPA-Cy5 and PDPA-Cy7.5 (molar ratio, 9:1) in near-infra-red window was selected for quantitative imaging of internalisation in vivo, and BiRN comprised of PDPA-BDP and PDPA-Cy3.5 (molar ratio, 6:4) in visible window was used as an alternative for in vitro and intravitral microscopnic imaging studies (Supplementary Table 2).

Transmission electron microscopy (TEM) and dynamic light scattering (DLS) analyses showed that the BiRN was well-dispersed spherical structure with a diameter of 25.6 ± 2.2 nm at pH 7.4 and dissociated into unimer with a diameter of 7.6 ± 1.1 nm at pH 5.4 (Fig. 2b, c and Supplementary Fig. 6a, b). Fluorescence images and spectra of BiRN in buffers with different pH demonstrated the excellent functions of each module: ‘OFF-ON’ module had a pH transition of 6.28, a sharp pH response (ΔpHₚHOFF = 0.21), and high fluorescence activation ratio of 111-fold; ‘always-ON’ module presented a constant fluorescence signal (F₀max/F₀min = 1.03) in a broad pH range (Fig. 2d, e and Supplementary Table 2). In contrast, the fluorescence signals of two modules only exhibited negligible fluctuations towards other substances, demonstrating the high specificity of BiRN towards pH variations (Supplementary Fig. 7). The normalised ratiometric signals of the ‘OFF-ON’ to ‘always-ON’ module intensities (O/A) of BiRN close to 0 at tumour extracellular pH, whereas turn to 1 at endocytic organelle pH (Fig. 2f and Supplementary Fig. 6e), demonstrating the BiRN can be exploited to quantify cellular internalisation of nanoparticle. BiRN nanoparticles keep stable in undiluted mouse plasma over 24 h incubation as indicated by the slight change of fluorescence ratio (Supplementary Fig. 6f).

BiRN quantifies nanoparticle internalisation in vivo. For in vivo quantification of internalisation, BiRN nanoparticles (20 mg kg⁻¹) were administrated into mice bearing 4T1 breast tumours by intravenous injection. As early as 10 min post-injection, the BiRN nanoparticles rapidly accumulated into the tumour sites as indicated by the observable Cy7.5 signal, while no significant nanoparticles were endocytosed by cells within tumours due to the undetectable Cy5 and O/A signals, revealing the negligible internalisation of accumulated nanoparticle at early time-points. The Cy7.5 signals increased continuously over 24 h due to the passive accumulation of BiRN into tumour tissues through EPR effect (Fig. 3a). The Cy5 signal also increased gradually in tumour tissues with high tumour to normal tissue (T/N) contrast (Fig. 3b), indicating the efficient internalisation and activation of nanoparticle inside cells. The dual-channel fluorescence images were further collected for 7 days. The fluorescence signals of both channels gradually decreased after 48 h post-injection of BiRN, which probably due to the slow clearance of nanoparticles from mice (Supplementary Fig. 13a, b). Accordingly, O/A signal reached a maximum at 12 h post-injection followed by a slight decrease in the subsequent monitoring period (Supplementary Fig. 13c).

To quantify the nanoparticle internalisation, the calibration curve between O/A signal and internalisation percentage of the BiRN was established. A plot of O/A as a function of turn ‘ON’ (i.e. internalisation) percentage presented a linear standard curve in a concentration-independent manner (Fig. 3c, d and Supplementary Fig. 14a), which allows for quantifying internalisation efficiency regardless of nanoparticle concentrations.
We further successfully established the individual standard curves ($R > 0.9500$) for main organs, such as the heart, lung, spleen, liver, kidney, tumour and muscle (Supplementary Fig. 14b), allowing the quantitative measurement of internalisation in each organ.

We then quantified the real-time nanoparticle internalisation using the established standard curves. As shown in Fig. 3e, the tumour endocytosis percentage reached a maximum at 12 h post-injection (~17.5%). The time-dependent tumour accumulation of nanoparticles was also determined by fluorometry. Based on the
Fig. 3 Real-time monitoring of BiRN internalisation in vivo. a 4T1 tumour-bearing mice were administrated intravenously with BiRN (20 mg kg$^{-1}$), dual-channel fluorescence images were captured at selected time-points. Insets are magnified images. O/A channel images were generated from the ratio of Cy5 to Cy7.5 by ImageJ software. b Time-dependent Cy5 and Cy7.5 fluorescence intensity after BiRN injection. Mean ± s.d. (n = 4 biologically independent mice). c Establishment of the calibration curve for quantification of BiRN internalisation. Above: A diagram to illustrate the sample and calculation formula used for the preparation of calibration curve. Below: Representative fluorescence images of BiRN with different turn ON percentages. d O/A signal as a function of turn ON percentage. The polymer concentration is 50 μg mL$^{-1}$. Mean ± s.d. (n = 3 biologically independent experiments). e Time-dependent endocytosis percentage of BiRN in tumour tissues after nanoparticle injection. Mean ± s.d. (n = 4 biologically independent mice). f Time-dependent intratumoural accumulation and endocytosis amount after BiRN injection. Mean ± s.d. (n = 4 biologically independent mice). g The fluorescent images of excised tumour and major organs at 24 h post-injection of BiRN. h The O/A images in six different tumour models (lung, pancreatic, breast, head & neck and PDX esophageal cancers). White arrows represent tumours. i Interstitial tumour pH (pHe) of different tumour types used for in vivo imaging. Mean ± s. d. (n = 3 biologically independent mice for A549 and HN5 models; n = 4 biologically independent mice for other models). j Heat map (n = 4 biologically independent mice) shows the heterogeneity of nanoparticle accumulation amount (left), endocytosis percentage (middle) and endocytosis amount (right) between various tumour models.
internalisation efficacy and accumulation results, the real-time internalised nanoparticles in tumours were successfully obtained (Fig. 3f). Results showed that about 15.5% of intratumoural nanoparticles (ca. 1.74% ID/g), that is 0.27% ID/g of nanoparticles, were internalised into the cells within tumour tissues at 24 h. The ratiometric imaging of excised organs demonstrated that nanoparticles internalisation in tumour mass is much efficient than in other organs/tissues (Fig. 3g and Supplementary Fig. 15). Despite most of the nanoprobe trapped in the monocellular phagocyte system (i.e. liver and spleen), the internalisation efficiency of BiRN in liver and spleen was only 2.6 and 5.3%, respectively (P < 0.01). The tumour internalisation of BiRN was further investigated in the dose escalation study. We found the efficiency of nanoparticle internalisation into tumour tissues decreased at a high dose of 40 mg kg$^{-1}$ probably due to the saturation of nanoparticle internalisation (Supplementary Fig. 16).

To explore the broad application in quantification of nanoparticle internalisation, we established six animal tumour models, including orthotopic HN5 head and neck cancer, multiple subcutaneous cancer (breast, pancreatic, lung) and patient-derived xenograft (PDX) oesophageal carcinoma models.

In all six tumour models, we successfully quantified the BiRN internalisation in solid tumours and other organs/tissues (Fig. 3h and Supplementary Figs. 17–22). To elucidate the impact of the acidic extracellular tumour microenvironment (pH$_e$) on the stability of BiRN, the pH$_i$ of interstitial fluid in all tumour models was determined by needle microelectrode protocol (Fig. 5b and Supplementary Fig. 24). We found that the average pH$_i$ value is 6.78 ± 0.20, none of the tumour interstitial pH$_e$ below the pH$_i$ of BiRN that guaranteed the exclusive activation inside cells, permitting the accurate measurement of nanoparticle internalisation in solid tumours (Fig. 3i). Heat map demonstrated the heterogeneity of BiRN accumulation and endocytosis within and between tumour types (Fig. 3j). Only 10.7–28.2% of the intratumoural nanoparticles were internalised over 24 h post-injection. The tumour distribution studies showed that 0.50–2.56% ID/g of administered nanoparticles accumulated in the tumour sites. Based on distribution data, we successfully quantified that only 0.09–0.72% ID/g of nanoparticles were sequestered by intratumoural cellular compartments.

BiRN profiles the intracellular micro-distribution of nanoparticles. To further investigate the microscopic internalisation of nanoparticle in living tumour-bearing mice, 4T1 tumours grown in dorsal window chambers were established to assess the function of BiRN in vivo by dual-photon intravital fluorescence microscopy. At 3 h post-injection of BiRN, the ‘always-ON’ signal distributed throughout the tumour microcirculation homogenously and extravasated in the tumour extravascular spaces, while the ‘OFF-ON’ signal was only colocalized with dot-shaped ‘always-ON’ signal in the extravascular region (Fig. 4a, b and Supplementary Movie 3). Ratiometric processing removes all the non-internalised fractions and leaves only the internalised hot-spots for accurate counting (Fig. 4c). We determined that 15.6 ± 1.7% of the homing nanoparticles were internalised by 3 h (Fig. 4d), consistent with the in vivo tumour results in Fig. 3a (12.5 ± 4.9%, 3 h).

After the animal imaging, the excised tumours were collected for tissue sectioning. The fluorescence imaging of the whole-mount tumour sections (Fig. 4e) displayed an intra-individual heterogeneity in microscopic distribution and internalisation of intratumoural nanoparticles. The spatial pattern of the internalised nanoparticles in the tumours was found significantly different from that of the homing particles, exhibiting loss of the diffuse signal and leaving the highlighted punctate pattern after ratiometric processing. These data strongly support our central hypothesis that BiRN design enables the differentiation of extracellular and intracellularly located nanoparticles for accurate counting of endocytic events in living animals.

BiRN internalisation predicts therapeutic efficacy of nanomedicines. Real-time imaging of drug efficacy and toxicity has shown great potential for the evaluation of treatment response and biosafety. To investigate whether quantification of BiRN internalisation can help to predict nanotherapeutic efficacy, we introduced a pH/cathepsin B hierarchical-responsive nanomedicine recently reported by our group. The nanomedicine was prepared through self-assembly of PEG-b-PDPA polymer-paclitaxel conjugate with enzyme cleavable Gly–Phe–Leu–Gly (GFLG) tetrapeptide linker (PEG-b-P(DPA-r-PTX), also abbreviated as PDPA-PTX). In our hypothesis, PDPA-PTX keeps micelle state in tumour microcirculation and extravascular space, but rapidly dissociated in early endosome once being internalised. After being translocated into lysosomes, the tetrapeptide linker can be cleaved by cathepsin B and the released PTX was transported into the cytoplasm, leading to effective tumour suppression (Fig. 5a).

To determine whether intracellular exposure of nanomedicine is the prerequisites for its anti-tumour efficacy, we performed the mechanism study of nanoparticle uptake. The results demonstrated that BiRN was endocytosed by 4T1 cells through caveolin- and dynamin-dependent endocytosis. Dynasore treatment reduced the uptake of BiRN by ~90% (Supplementary Fig. 24). The O/A images confirmed dynasore can dramatically suppress the intracellular exposure of nanoparticle (Fig. 5d). We next sought to examine the in vitro anti-tumour efficacy of PDPA-PTX. The results showed the efficacy of PDPA-PTX (IC$_{50}$ 1.25 μM) was comparable to that of free drug (IC$_{50}$ 0.92 μM) on a per PTX basis. In contrast, dynasore treatment led to a ~90% decrease in anti-tumour efficacy of PDPA-PTX (IC$_{50}$ 12.87 μM) due to the marginal uptake of PDPA-PTX (Fig. 5e).

We next evaluated the potential of BiRN to predict disease progression after treatment with PDPA-PTX nanoparticle. For in vivo correlation analysis, LC-MS was performed to quantify total and released PTX contents within the tumour mass. We found that intratumoural total level of PTX is 1.80 ± 0.4% ID/g of administered PDPA-PTX, which is comparable to that of BiRN (1.74% ID/g). Furthermore, a good correlation was observed between fluorescence intensity of OFF-ON module and the released PTX content (R = 0.8304, P = 0.0008). Results demonstrated the feasibility of using intracellular nanoparticle exposure (Cy5 signal) as surrogate indicator for intracellular drug bioavailability (Fig. 5f). To test whether intracellular nanoparticle exposure could help to estimate anti-tumour efficacy of nanomedicines, mice bearing orthotopic 4T1 tumours (n = 24) underwent in vivo imaging at 24 h post-administration of BiRN (Fig. 5g). Fluorescence images showed endocytosis and accumulation varied vastly among individuals (Fig. 5h and Supplementary Fig. 26a). We stratified mice into low- and high-endocytosis groups according to the intracellular nanoparticles level (Fig. 5h, i).
PDPA-PTX nanoparticle was administered intravenously after imaging, and tumour progression was monitored every other day (Fig. 5j and Supplementary Fig. 25). Comparing with mice in the control group with 400% change in tumour volume at day 10, the mice subjected to chemotherapy had slower tumour growth (low group, 103.9%; high group, 44.5%). Moreover, we observed significant differences ($P = 0.026$) in percentage of tumour volume change among the low- and high-endocytosis groups 2 days after treatment with PDPA-PTX, and the differences were even more significant ($P = 0.002$ at day 10) with time lapse (Fig. 5j). A good correlation ($R = 0.8636$, $P < 0.001$) between the level of intracellular nanoparticle and tumour inhibition percentage was observed on day 16 (Fig. 5k), which implies BiRN is a suitable indicator for the prediction of therapeutic efficacy of PDPA-PTX in individual subjects. In addition, the proportion of mice with lung metastases in the high group was also lower than that in low group (16.7% vs. 58.3%, Fig. 5l, m). Based on the Cy7.5 signal, we re-stratified mice bearing 4T1 tumours into low- and high-accumulation groups. Unfortunately, nanoparticle accumulation is not a good indicator for the prediction of therapeutic outcome (Supplementary Fig. 26). These results demonstrated that intracellular exposure of BiRN is a good indicator for the prediction of intracellularly bioavailable nanomedicine in tumour mass.

We proceeded to test the ability of BiRN to predict the therapeutic efficacy of other nanomedicines. PEGylated doxorubicin liposome (Doxil) was chosen as model nanomedicine for further experiments. We first performed the correlation analysis...
Fig. 5 BiRN predicts therapeutic efficacy of PDPA-PTX nanoparticle. a The design of PDPA-PTX and schematic of internalisation and PTX release of PDPA-PTX in cells. b TEM images of PDPA-PTX at pH 7.4 and 5.4. Polymer concentration, 1 mg mL\(^{-1}\). c Intratumoural levels of released PTX at 48 h post-injection of BiRN and PDPA-PTX (PTX: 10 mg kg\(^{-1}\)). d Coimats of 4T1 cells treated with BiRN (100 μg mL\(^{-1}\)) with or without dynasore. e Cytotoxicity of free PTX and PDPA-PTX micelle. Mean ± s.d. (n = 6 biologically independent experiments). f Correlation between intracellular nanoparticle exposure and intratumoural levels of released PTX at 48 h post-injection of BiRN and PDPA-PTX (PTX: 10 mg kg\(^{-1}\)). g In vivo PTX release profiles of PDPA-PTX in different conditions. Mean ± s.d. (n = 3 biologically independent experiments). h Confocal images of 4T1 cells treated with BiRN (100 μg mL\(^{-1}\), scale bar, 100 nm. i In vitro PTX release profiles of PDPA-PTX in different conditions. Mean ± s.d. (n = 3 biologically independent experiments). j In vitro PTX release profiles of PDPA-PTX in different conditions. Mean ± s.d. (n = 3 biologically independent experiments).
of intracellular BiRN nanoprobes and Doxil. After sequential injection of BiRN and Doxil to 4T1 tumour-bearing mice, flow cytometry analyses of cell suspensions from tumour tissues provided conclusive evidence that good correlation ($R = 0.7996$, $P < 0.001$) was observed between Cy5 and doxorubicin signals at single-cell level (Fig. 6a). Next, we evaluated the potential of BiRN to predict disease progression after treatment with Doxil (Fig. 6b). Fifty mice bearing orthotopic 4T1 tumours were imaged after intravenous administration of BiRN to measure total tumour accumulation and endocytosis (Fig. 6c). Doxil was administered intravenously after imaging, and tumours progression was monitored every other day (Supplementary Fig. 27a). We observed the relative tumour volume was linearly correlated ($R = 0.7251$, $P = 0.0022$) with endocytosis amount rather than accumulation (Fig. 6d and Supplementary Fig. 27b). We stratified mice into low, medium and high groups according to intracellular nanoparticle exposure. Similarly, we found that the high group had significantly ($P = 0.03$) better growth inhibition than low group (Fig. 6e). Therefore, BiRN reporter can be served as an effective predictor for the therapeutic outcomes of other nanomedicines.

**Discussion**

We have described a two-modular nanoreporter technology for quantitative imaging of cellular internalisation in living animals. This BiRN reporter successfully converts the subtle pH change involved in the endocytic events ($\Delta$pH $< 0.6$) into digitised ratiometric signal output, permitting the precision quantification of cell internalisation in vivo. While, conventional pH sensors can chemically resolve continuous variations during the endosome maturation process, but fail to accurately report the endocyctic events. The present nanoreporter was successfully engineered with several critical functions that enable the directly gated counting of nanoparticle internalisation in living subjects without any sample processing. Firstly, positive cooperativity in the self-assembly of pH-responsive block copolymer drives the sharp pH response ($\Delta$pH$_{ON/OFF}$ $< 0.25$), which is essential for chemically differentiating the extracellular and endocytic pH. Secondly, the ultra-fast disassembly (<5 ms) of the nanosystem enables the real-time recording of the endocytic events without any lag time. Thirdly, our design exploits pH differences between acidic tumour microenvironment and early endosome, which is a recognised hallmark of cell internalisation regardless of tumour types. Moreover, fluorescence imaging provides a real-time, noninvasive, convenient and lower-cost paradigm for quantitative measurement of the intracellular bioavailability of nanomedicines.

Our studies revealed that only 20% (median) of the nanoparticles resided in solid tumours were internalised into the cell components, while ~80% of the nanoparticles were sequestered in the acellular regions of the tumours at 24 h. More importantly, our BiRN technology demonstrated that intracellular nanoparticle exposure exhibited a strong correlation with anti-tumour efficacy of nanomedicines, whereas nanoparticle accumulation in tumour mass presented a poor correlation with therapeutic nanomedicine efficacy due to the high heterogeneity of internalisation efficiency between individual subjects. Hence, quantifying the intracellular bioavailability of nanomedicines rather than the overall accumulation in solid tumours is a suitable indicator for the prediction of anti-tumour efficacy and patient stratification for personalised treatment.

In summary, we successfully quantified the internalisation of nanoparticles that accumulated in the solid tumours at whole animal, intravital, excise tissue and tissue section levels by the
BiRN technology. This internalisation nanoprober offers a powerful tool to predict anti-tumour efficacy of different types of nanomedicines by patient stratification based on intracellular exposure of nanoparticle in tumour mass. This nanotechnology-enabled paradigm also advances a valuable tool to evaluate the tumour-targeting efficacy of new targeted nanomedicines, to uncover the dynamic Nano–Bio interaction in solid tumours.

**Methods**

**Materials.** The N-hydroxysuccinimidyl ( NHS) esters of different fluorescent dyes were purchased as follows: BDP, Cy3.5, Cy5 and Cy7.5 from Lumiprobe Corp.; IC-Gel (ICG) from Luminos Technologies. Polyethylene glycol dimethyl ether phosphate (PEG-DMA) and 2-aminoethyl methacrylate (AMA) were purchased from Polysciences Company. Initiator V65 was purchased from Yuanye Biotechnology Co. Ltd. Other reagents and solvents were from Thermo Fisher Scientific or Sigma-Aldrich Inc.

**Syntheses**

**Syntheses and characterisation of dye-labelled block copolymers.** PEG-b-PDPA-AMa copolymer was synthesised by atom transfer radical polymerisation (ATRP) methodology10 using ATRP initiators PEG-DMA (8 mmol) and PMDETA (0.1 mmol) were charged into a reaction flask. Then, the above substances were dissolved by the addition of the mixed solvents of 2-propanol (2 mL) and N,N-dimethylformamide (DMF, 2 mL), the flask was sealed and subjected to vacuum for 12 h at 40 °C. After the reaction, the reaction mixture was diluted with 10 mL tetrahydrofuran (THF), and the catalyst was removed by neutral aluminium oxide column. Most of the eluent was removed by rotovap. The residue was dialysed in distilled water for 24 h and then lyophilised to obtain white powder. The purified PEG-b-PDPA-AMa copolymer was characterised by 1H 400 MHz NMR and gel permeation chromatography (GPC). Molecular weight (Mn and Mw) and PDI of PEG-b-PDPA-AMa were 22.27, 27.39 and 1.23kDa, respectively.

For fluorescent labelling of the copolymer, the dye-containing DMF solution (10 mg mL−1) was added into a solution of PEG-b-PDPA-AMa copolymer in DMF (50 mg mL−1). Then, the solution was stirred overnight in the dark. The purified PEG-b-PDPA-Dye, copolymers were obtained by preparative GPC to remove the unconjugated dye molecules. The resultant dye-conjugated polymers were lyophilised and kept at −20 °C until further use. The conjugation efficiency of the dye to each polymer was determined using UV–Vis spectrophotometry. The molar feed ratios of different dyes to AMa and corresponding conjugation ratios were listed in Supplementary Table 1.

**Characterisation of various monochrome nanoprobes.** The fluorescence emission spectra of nanoprobes in PBS buffers with different pH were measured by a Confocal Laser Scanning Spectrometer (Carl Zeiss, Germany). Table 1 shows the results for Cy5-b-PDPA-Dye nanoprobe for example, the nanoparticle stock solution was diluted to 0.1 mg mL−1 with PBS buffer of different pH. The samples were excited at 630 nm with a slit width of 5 nm, and emission spectra were collected from 650 to 720 nm. The peak intensity of emission spectra was used for the quantitative assessment of the pH-sensitivity properties. The value of R5 = FpH/FpH = pH, where FpH and FpH = pH are shown in Supplementary Table 1. The excitation and emission wavelength (λex,λem) of dyes used to quantify pH-sensitivity are as follows: BDP (488/500–600 nm), Cy3.5 (575/ 590–700 nm), BDPES (630/650–750 nm), Cy5 (630/650–750 nm), Cy7.5 (780/ 795–900 nm) and ICG (780/795–900 nm).

**Preparation and characterisation of BiRN nanoprobes.** After fluorophores screening of ‘OFF-ON’ and ‘always-ON’ modules among monochrome nanoprobes. Two dyes (BDP, Cy5) were selected as fluorophores of ‘OFF-ON’ module due to their high activation ratios (R0 ≈ 100). Accordingly, four dyes (Cy3.5, BDPES, Cy7.5 and ICG) with low activation ratio (R0 ≤ 10) were chosen as potential ‘always-ON’ fluorophores. Subsequently, a series of hybrid nanoparticles comprised of ‘OFF-ON’ modules and potential ‘always-ON’ modules with different molar ratios were prepared as mentioned above. Followed by fluorescence characterisation, the hybrid nanoparticles were diluted to 0.1 mg mL−1 in phosphate-buffered saline (PBS, 100 mM) with different pH from 5.4 to 7.2 (with 0.2 pH interval). The samples were successively excited with two excitation wavelengths to test the fluorescence emission spectra of ‘OFF-ON’ and ‘always-ON’ modules on a fluorescence spectrometer (Cary Eclipse, Agilent, USA).

Transmission electron microscopy (TEM) (EM 1200EX, Hitachi, Japan) was used to visualise the morphology of nanoparticles at different pH. The nanoparticles were diluted with Milli-Q water to 1 mg mL−1 or hydrochloric acid solution (pH = 5.4), and then negatively stained by 1% phosphotungstic acid. The TEM images were captured on a transmission electron microscope ( TEM-1400, JEOL, Japan).

Fluorescent images of nanoprobe solution (100 µg mL−1) at various pH values were acquired on an IVIS in vivo imaging system (Lumina Series III, PerkinElmer, USA). After auto-exposure with an IVIS fluorescence characterisation, the fluorescence intensity ratio for the acceptor to donor or ‘OFF-ON’ module to ‘always-ON’ module over time was used for the stability evaluation.

**Preparation and characterisation of PDDA-PTX micelle.** Firstly, PDDA-PTX micelle comprised of PEG-b-PDPA-Ptx was prepared as mentioned above. PTX release from PDDA-Ptx micelle was performed by incubation of the micelle in McIlvaine’s buffer (pH 6.0) at 37 °C in the presence of papain (5.0 µM) for 48 h. The stability of PDDA-Ptx micelle in PBS buffer (pH 7.4) was monitored for 24 h at 37 °C. At pre-determined time-points, the released PTX amount in samples was analysed by HPLC (Agilent, USA) at UV wavelength of 230 nm. An Agilent SB-C18 (4.6 × 250, 5 µm) was maintained at 40 °C and a flow rate of 1 mL min−1 was used, with a mobile phase (30% acetonitrile, 70% water) at a flow rate of 20 µL min−1.

**Preparation of pegylated liposomal doxorubicin.** Liposomes were prepared by the traditional thin film–hydration method and DOX was loaded into the liposomes via the reverse loading with an ammonium sulfate gradient. Briefly, HSPC, Chol and DSPS–mPEGn (molar ratio = 10:5:1) were dissolved in trichloromethane and subsequently the solvent was removed by rotovap at 37 °C to form a thin lipid film. The resulting film was hydrated with ammonium sulfate solution (123 mM), followed by sonication for 30 min. The prepared liposomes were passed through a Sephadex G-50 column equilibrated with PBS buffer (10 mM, pH = 7.2) to replace the liposomal internal solution. The DOX-HCl was added into the eluted liposome suspension and incubated at 70 °C for 30 min. The separation of liposome from unloaded DOX was performed by chromatography using a Sephadex G-50 column. The concentration of DOX was determined at 495 nm utilising ultraviolet–visible (UV–Vis) spectrophotometer (UV3000, Hitachi, Japan) after lysis of liposomes with Triton X-100.
In vitro studies

Cell culture. 4T1 and MCF-7 breast carcinoma, A549 lung cancer cells, Panc02 and BxPC-3 pancreatic cancer cells, RAW264.7 macrophage and NIH3T3 fibroblast were obtained from National Infrastructure of Cell Line Resource. HNS5 head and neck cancer cell line was established from a head and neck cancer patient and provided by Dr. Jinning Gao Lab at University of Texas Southwestern Medical Centre. Above cell lines were maintained in complete medium (RPMI 1640 or DMEM with 10% FBS and antibiotics) in 5% CO2-containing humidified atmosphere at 37°C.

Real-time live cell imaging. The cells were seeded in glass-bottom dishes and incubated for 24 h at 37°C, 5% CO2 atmosphere to allow cell adhesion and growth. For real-time imaging, cell nuclei were counterstained with Hoechst 33342 (5 μg/mL) firstly and washed with PBS. Subsequently, confocal images were captured by a confocal microscope (A1R-Storm, Nikon, Japan) provided by Dr. Jinming Gao Lab at University of Texas Southwestern Medical Centre. Above cell lines were maintained in complete medium (RPMI 1640 or DMEM with 10% FBS and antibiotics) in 5% CO2-containing humidified atmosphere at 37°C.

Establishment of the calibration curve for quantification of BiRN internalisation. For the establishment of the calibration curve, BiRN diluted in the medium with pH 7.4 and pH 5.4 were used to simulate nanoprobes with micelle state in extracellular matrix and with unimer state in endocytic organelles, respectively. Taking PBS buffer as medium, the nanoparticles with constant polymer weight were diluted separately in two kinds of pH buffers (50 μL each) to pre-calculated concentrations, and the samples with varying ‘turn on’ (endocytosis) percentages (proportion of nanoparticles in pH 5.4) were obtained. The samples with 0, 5, 10, 20, 30, 40, 50 and100% endocytosis percentages were prepared, and then placed in a black 384-well plate, followed by fluorescence imaging using an IVIS in vivo imaging system (Lumina Series III, PerkinElmer, USA). Fluorescence images were captured using auto-exposure with band-pass filters (λex/λem: 620 ± 10 nm/670 ± 20 nm for Cy5 and 780 ± 10 nm/845 ± 20 nm for Cy7,5). The mean fluorescence intensities of two channel images with varying endocytosis percentages were measured using Living Image Software (PerkinElmer). The ratio’s value as a function of endocytosis percentage is plotted according to the following formula:

\[ \text{Ratio} = \frac{F_{\text{Cy5, pH 4}} + F_{\text{Cy5, pH 4}}}{F_{\text{Cy7, pH 4}} + F_{\text{Cy7, pH 4}}} \]

To establish a calibration curve under in vitro physiological condition, a series of tissues homogenates with two pH were prepared to dilute BiRN. Fresh tissues including the heart, lung, spleen, liver, kidney, tumour and muscle were collected immediately after tumour-bearing mice were transcardially perfused with 20 mL PBS buffer. The excised tissues were cut into small pieces, weeded out connective tissues, weighed and homogenised in PBS with pH 7.4 or pH 5.4 using a homogeniser (T10 basic Ultra-Turrax, IKA, Germany). The samples of tissue homogenates with varying endocytosis percentages were prepared following the aforementioned dilution method and imaged on an IVIS in vivo imaging system. The calibration curve of each tissue homogenate was used to calculate corresponding endocytosis percentages. BiRN was diluted in PBS and tumour homogenates with varying endocytosis percentages were imaged on the fluorescent spectrometer (F-7000, Hitachi, Japan). Then, a linear relationship between fluorescence intensity and concentrations was established using the linear regression method. For intratumoural nanoparticle accumulation quantification, tumours (n = 4) were dissected at pre-determined time-points post-injection of nanoparticle. The homogenates and centrifugal supernatant of tumours were prepared by the above-mentioned methods. The amount of intratumoural nanoparticles were calculated from the linear standard curves.

In vivo studies

Animal models. All animal care and procedures were carried out in compliance with ethical compliance and approval by the Animal Ethics Committee of Peking University. Female nu/nu nude, BALB/c and NOD SCID mice (18-22 g) were purchased from Vital River Laboratory Animal Centre (China). Animals were housed in groups of 4–5 per cage, maintained at a temperature of ~25°C in a humidity-controlled environment with a 12 h light/dark cycle. The BALB/c mice were subcutaneously innoculated with 4T1 tumour cells (1 × 106 cells per mouse) on their right flanks. For tumour accumulation and cellular endocytosis, BiRN was administrated intravenously into other six tumour models mentioned above. Then, the mice were monitored by sequential acquisition at 670 and 845 nm at designated time-points. Mice were euthanised after the last imaging session, followed by resection of the tumour and organs. The dissected tumours and organs were fluorescently imaged immediately. The images of Cy5 and Cy7.5 channels were extracted using Living Image Software (PerkinElmer) and the ratiometric images were generated by Imagej software (NIH).

Intratraductal imaging of BiRN nanoprobes distribution in vivo. For visualisation of nanoprobe distribution in living animal, female nude mice (20–22 g) were implanted with a dorsal skin flap chamber following the method previously published15. 4T1 tumour cells (1 × 106) were harvested in up to 20 mL of phenol red-free culture medium and injected between the dermis and the exposed fascia.
Over weeks, tumours reached several millimetres in size and became vascularised. Intravital imaging was performed on a dual-photon fluorescence microscope (TCS-SP8, Leica, Germany). Mouse brains were anesthetized with isoflurane (1.5%, v/v) on a heated mouse warming plate. Intravenous injection was performed using a 19G needle. Two days after injection, 20 µL of the dye solution was injected into the right carotid artery. After injection, the dye concentration in the tumour and surrounding tissues was imaged using confocal microscopy. The experiments were repeated three times with similar results and a series of representative images from each group were shown. Intravital imaging of tumours post-injection of BiRN was performed three times using biologically independent mice with similar results, and a series of representative images were shown. For the whole-mount imaging analysis of BiRN and internalisation for tumour slices, the experiment was repeated three times with similar results, a series of representative images were shown.

**Statistics and reproducibility.** TEM experiments of BiRNs and PDPA-PTX nanoparticles in different pH solution were repeated three times independently with similar results, and one representative image from each group was shown. Confocal imaging of different cells incubated with BiRN in the absence or presence of Dynasore and pulse-chase imaging of BiRN in different cells were repeated at least three times with similar results, and a series of representative images from each group were shown. Intravital imaging of tumours post-injection of BiRN was repeated three times using biologically independent mice with similar results, and a series of representative images were shown. For the whole-mount imaging analysis of BiRN distribution and internalisation for tumour slices, the experiment was repeated thrice with similar results, a series of representative images were shown.

**Data availability**

The source data underlying Fig. 4d and all the mouse intervention studies are provided as a Source Data file. All the other data supporting the findings of this study are available within the Article and its Supplementary Information files and from the corresponding author upon reasonable request. A Reporting Summary for this article is available as a Supplementary Information file. Source data are provided with this paper.

Received: 1 December 2020; Accepted: 23 March 2021; Published online: 22 April 2021

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**Acknowledgements**

This work is financially supported in part by the National Key Research and Development Program of China (2017YFA0205600), the National Natural Science Foundation of China (NSFC) grants (81973260 and 81671817) and Beijing Natural Science Foundation (JQ19025). We thank Bo Xu and Wenzhe Li for assistance with cell and animal imaging.

**Author contributions**

Q.Y. and Y.G.W. are responsible for all phases of the research; B.C. assisted with the experimental design; A.P. and Z.W. helped with syntheses and characterisation of different dye-conjugated polymers; M.T., H.X. and X.Y. helped with in vitro cell studies, establishment of calibration curves and in vivo imaging; Y.Y assisted with data analysis; Y.Q.W. provided window chamber model and assisted intravital imaging; W.C. assisted with fluorescence measurement and quantitative analysis by LC-MS; H.D. and M.C. helped with syntheses and characterisation of PTX-conjugated polymers. Y.N.W. and Z. L. provided the PDX model. Y.Q. wrote the manuscript. B.C., Q.Z. and Y.G.W. revised the final draft. All authors read, provided feedback on and approved the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22678-z.

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**Peer review information** *Nature Communications* thanks Xiaoyuan Chen and Kanji Pu for their contribution to the peer review of this work. Peer reviewer reports are available.

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