Intratumor mapping of intracellular water lifetime: metabolic images of breast cancer

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Shutter-speed pharmacokinetic analysis of dynamic-contrast-enhanced (DCE)-MRI data allows evaluation of equilibrium inter-compartmental water interchange kinetics. The process measured here – transcytosomal water exchange – is characterized by the mean intracellular water molecule lifetime ($\tau_i$). The $\tau_i$ biomarker is a true intensive property not accessible by any formulation of the tracer pharmacokinetic paradigm, which inherently assumes it is effectively zero when applied to DCE-MRI. We present population-averaged in vivo human breast whole tumor $\tau_i$ changes induced by therapy, along with those of other pharmacokinetic parameters. In responding patients, the DCE parameters change significantly after only one neoadjuvant chemotherapy cycle: while $K_{\text{intra}}$ (measuring mostly contrast agent (CA) extravasation) and $k_{\text{ep}}$ (CA intravasation rate constant) decrease, $\tau_i$ increases. However, high-resolution, (1 mm)$^2$, parametric maps exhibit significant intratumor heterogeneity, which is lost by averaging. A typical 400 ms $\tau_i$ value means a trans-membrane water cycling flux of $10^{13}$ H$_2$O molecules s$^{-1}$/cell for a 12 mm diameter cell. Analyses of intratumor variations (and therapy-induced changes) of $\tau_i$ in combination with concomitant changes of $\nu_e$ (extracellular volume fraction) indicate that the former are dominated by alterations of the equilibrium cell membrane water permeability coefficient, $P_w$, not of cell size. These can be interpreted in light of literature results showing that $\tau_i$ changes are dominated by a $P_w$(active) component that reciprocally reflects the membrane driving P-type ATPase ion pump turnover. For mammalian cells, this is the Na$^+$,K$^+$-ATPase pump. These results promise the potential to discriminate metabolic and microenvironmental states of regions within tumors in vivo, and their changes with therapy. © 2014 The Authors. NMR in Biomedicine published by John Wiley & Sons Ltd.

Keywords: maps; intratumor; heterogeneity; metabolic activity; therapy

INTRODUCTION

Tumor heterogeneity

Cell type and metabolic heterogeneity are crucial tissue characteristics. For example, there is much current interest in intratumor phylogenetic diversity. Its metabolic consequences are likely of great significance in therapy resistance or tolerance (1–6). This heterogeneity presents a challenge to blood tests, point biopsies, and ex vivo tissue homogenization, and puts a premium on in vivo assessment with the highest possible spatial resolution and on an individual-by-individual basis (7). The most feasible current methods of human metabolic imaging, $^{18}$F

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Abbreviations used: AML, acute myeloid leukemia; CA, contrast agent; $CC_{\text{intra}}$, core ROIs/complete responder/therapy point $X$; $CC_{\text{non-intra}}$, core ROIs/non-complete responder/therapy point $X$; DCE, dynamic contrast enhanced; FXL, fast exchange limit; FXR, fast exchange regime; H&E, hematoxylin and eosin; IDC, invasive ductal carcinoma; IV, intravenous; $k_{\text{int}}$, CA intravasation rate constant; $K_{\text{intra}}$, CA extravasation transfer constant; L-NAME, N$\text{-}$nitro-L-arginine methyl ester; NACT, neoadjuvant chemotherapy; $NKA$, Na$^+$,K$^+$-ATPase; non-PCR, non-complete responder by pathology; pCR, complete responder by pathology; RCB, residual cancer burden; $R_{\text{NKA}}$, rim ROIs/complete responder/therapy point $X$; $R_{\text{NKA}}$, rim ROIs/non-complete responder/therapy point $X$; ROI, region of interest; SDs, standard deviations; SSP, shutter-speed paradigm; SRX, slow exchange regime; $\tau_{\text{intra}}$, mean intracellular water molecule lifetime; $P_w$, therapy point $X$; ULR, univariate logistic regression; $\nu_e$, extracellular, extravascular volume fraction.
positron emission tomography (8) and hyperpolarized $^{13}$C magnetic resonance spectroscopic imaging (9), are extremely informative, but generally have insufficient resolution (typically 5 and 7 mm, respectively) to clinically assay intratumor heterogeneity, and are costly. In contrast, water hydrogen proton ($^{1}$H$_2$O) MRI affords millimeter or sub-millimeter spatial resolution in human studies, uses no ionizing radiation, is minimally invasive, and is relatively inexpensive. Though millimeter or sub-millimeter does not match the resolution of histopathology microscopy, it detects considerable intratumor heterogeneity. Conventionally, however, $^{1}$H$_2$O MRI is thought to provide only anatomical/vascular (and sometimes tissue functional) information. Fortunately, a new opportunity is presenting itself. It arises from the ability of MR to measure intercompartmental water molecule exchange kinetics. This study investigates the implications of the heterogeneity of such trans-cytoskeletal kinetics within malignant human breast tumors before and after neoadjuvant chemotherapy (NACT).

BACKGROUND

Mean intracellular water molecule lifetime ($\tau_i$)

The kinetics of the equilibrium (steady-state) exchange of water molecules across cell membranes have been studied by isotope labeling techniques for almost 60 years (reviewed in (10)) and by NMR methods for over 40 years (reviewed in (11–13)). For a ‘well-mixed’ cytoplasm, the kinetics can be expressed in Equation [1]:

$$\tau_i^{-1} = P_W (A/V)$$

where $\tau_i$ is the mean lifetime of a water molecule inside the cell, $P_W$, the cell membrane water permeability coefficient, and $(A/V)$ the claustrophobia ratio ($A$ is the individual cell surface area and $V$ the individual cell volume) (14). The NMR community tends to report $\tau_i$ values (11–13) while the isotope community gives $P_W$ values: $P_W$ is often labeled $P_x$, the diffusive permeability (10). (We note that, even though they have the same dimensions (distance/time), $P_W \approx P_x$ is not the same as $P_x$ the water permeability coefficient in the presence of a transmural osmotic gradient, which causes net trans-membrane water transport (flow) simultaneous with the much faster exchange (15–17).) Using Equation [1], one finds published $P_W$ and $\tau_i$ values to be in general agreement (13). The $\tau_i$ reciprocal, $\tau_i^{-1}$, is the unidirectional first-order rate constant, $k_{\text{in}}$, for equilibrium water efflux from the cell – there is no net transport (18). Values of $\tau_i^{-1}$ span four orders of magnitude – the extremes range from $10^{-2}$ s$^{-1}$ for Xenopus oocytes to $10^{2}$ s$^{-1}$ for erythrocytes (11–13,19). For virtually all other cells, however, $\tau_i$ values are hundreds of milliseconds and the ‘well-mixed’ approximation is quite good (20). For only the very largest, e.g. the Xenopus oocyte (1 mm diameter), does this condition just begin to fail (19).

Equation [1] can be approximated with Equation [3]:

$$\tau_i^{-1} = C (P_W/d)$$

where $C$ is a constant shape factor and $d$ the cell diameter. If the cell is spherically shaped, $C$=6 (if the cell shape is well approximated as a right cylinder, $C$=4). The quantity $d$ is a one-dimensional (1D) measure of cell size.

There has been some recent concern (21) that interpretation of literature data on the blood wash-out following bolus injections of isotopically labeled water implies cerebral cortical $\tau_i \geq 30$ s. This is almost two orders of magnitude greater than values generally found (10–13). Taking a typical value of $P_W = 1.4 \times 10^{-4}$ cm s$^{-1}$ (10), a $\tau_i$ of 30 s in Equation [3] yields a $C$ value of 250 $\mu$m: clearly much larger than the average cell (6). A simple tracer intravasation interpretation of the same blood wash-out data yields (22) a mean lifetime for extravascular water ($\tau_{\text{ex}}$) of 21 s – in good agreement with the blood capillary water permeability coefficient from isotopically labeled water experiments. Seemingly, $\tau_{\text{ex}}$ has been mis-assigned as $\tau_i$ in (21).

Active trans-membrane water cycling

The equilibrium water exchange process has been conceived as resulting from passive molecular mechanisms: simple diffusion across the lipid bilayer, passage through aquaporin membrane protein channels, leakage through membrane transporters, etc. (23–25). Changes in $d$ (or $C$) – cellular swelling or shrinking (edema) – will alter $\tau_i^{-1}$. However, the rate constant for $d$ change is at least an order of magnitude smaller than $\tau_i^{-1}$ (21). Any $\tau_i^{-1}$ changes not due to $d$ (or $C$) alterations must be due to changes in $P_W$, which has always been thought of as a passive cell membrane property ($P_W$ passive). However, NMR studies have recently revealed an active component ($P_W$ active) much larger than the passive contribution (17). This is due to active trans-membrane water cycling that accompanies active trans-membrane osmolyte cycling, which is paced by the driving cell membrane P-type ATPase ion pump (17). For mammalian cells, this is the Na$^+$,K$^+$-ATPase, NKA (17,26–28). The existence of this water cycling is significant.

NMR principles

The classic NMR measurements are mostly of homogeneous cell suspensions. The presence of an extracellular paramagnetic contrast agent (CA) increases the intrinsic longitudinal relaxation rate constant $R_{1o} = (\tau_i^{-1})$ of the outside water proton signal ($^{1}$H$_2$O$_o$). Though the term was introduced only in 1999 (29), this approach increases the longitudinal ‘shutter-speed’, $\tau_i^{-1} = | R_{1o} – R_{1i} |$, sufficiently that the water exchange NMR system is moved out of the fast-exchange-limit (FXL) condition ($R_{1i}$ is the intrinsic intracellular relaxation rate constant). A sufficient outside CA concentration, $[CA_{\text{ex}}]$, allows the NMR system to reach the slow-exchange-regime (SXR) condition. This is characterized by non-mono-exponential longitudinal magnetization recovery, but is distinct from the slow-exchange-limit and no-exchange-limit conditions (13,17,22,29,30). It is quite common to achieve the SXR with cell suspensions (11–13,17,22). However, if the $[CA_{\text{ex}}]$ value is only modest, and the system reaches only the fast-exchange regime (FXR) condition, then $\tau_i^{-1}$ measurement requires $\tau_i^{-1}$ variation by varying $[CA_{\text{ex}}]$ (17,22,29–31). The FXR condition features apparent mono-exponential longitudinal recovery, i.e., the measured $R_1$ is single valued, but has a non-linear $[CA_{\text{ex}}]$ dependence. In the FXL condition, this dependence is linear (13,17,22,29,30). (Sometimes, transverse $^{1}$H$_2$O relaxation is employed for cell suspensions (11,12).)

Dynamic-contrast-enhanced (DCE)-MRI

The spatial encoding of $^{1}$H$_2$O in MRI offers the possibility of measuring, and mapping, $\tau_i$ values in human biological tissues in vivo. A stepped CA infusion producing incremental plasma and interstitial CA steady-state levels works well in animal models (29), but is impractical for human use. However, in the common clinical DCE-MRI approach, serial $T_1$-weighted $^{1}$H$_2$O images are
obtained before, during, and after a bolus CA injection. The time-course of the CA bolus passage through the field of view is measured. The shutter-speed concept can be incorporated into the pharmacokinetic analysis of the time-course. This differs from the tracer pharmacokinetic paradigm, which is based on the fact that compartmentalization is not encoded in the tracer signal: it does not distinguish compartments entered by tracer. In DCE-MRI, CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continuously tracks the amounts of H$_2$O and CA in each compartment. Thus, imposition of a classic tracer analysis on DCE-MRI data joins these contradictory postulates about CA compartmentalization is intrinsic to the $^1$H$_2$O signal, which continues...
averaged before pharmacokinetic analysis. For (c) ‘image slice pixel-averaged’ data, the individual pixel time-courses are analyzed and then the resulting parameters averaged.

Parameter precision from Monte Carlo simulations

For the pCR subject selected for in-depth analyses, the precision of fitted DCE parameters ($\tau_i$ and $K_{\text{trans}}$) was estimated using a Monte Carlo approach detailed previously (48). The DCE-MRI time-courses for each tumor pixel in the chosen image slice, obtained at both TP0 and at TP1, were analyzed. Six fittings of each voxel DCE-MRI time-course were made, starting with randomly different initial sets of parameter values spanning broad ranges. The standard deviations (SDs) of the returned parameter values were calculated.

Histology

The response to NACT for each patient was determined by pathology analysis of post-therapy surgical specimens (after TP3) and comparison with pre-therapy biopsy specimens (before TP0). This comprised the determination of the residual cancer burden (RCB) and the relative change in tumor cell density using published methods (49,50). These analyses revealed that three patients were pCR – no cancer cells found in resection specimens – while the other eight were non-pCR – reduced cancer cell density in resection specimens compared with biopsy specimens. For the non-pCR case selected for in-depth analysis, a whole-mount slide of a slice of a biopsy core obtained pre-therapy (before TP0) was used to estimate the extent of necrosis and the cell densities in regions of the parametric tumor rim and tumor core. The biopsy core was obtained with a 14 gauge needle (1.6 mm ID), inserted from medial to lateral, and the hematoxylin and eosin (H&E)-stained slide was examined by microscopy at both low and high power (200x).

Statistical analyses

Tumor ROIs were drawn by experienced radiologists, who also measured the largest 1D tumor size according to the RECIST (Response Evaluation Criteria in Solid Tumors Group) (51) guideline. The results from pathology analyses were correlated with the MRI metrics using the ULR (univariate logistic regression) analysis in order to identify imaging biomarkers for early prediction of response and/or accurate assessment of residual disease following NACT.

RESULTS

Human breast cancer and therapy

We have presented many DCE time-courses of breast (13,18,46) and prostate (30,47) cancer data. Figure 1 displays whole breast axial DCE image slices obtained during CA passage for two subjects with IDC representative of the population here – one found a non-pCR (Fig. 1(a), (c); left breast) and one a pCR (Fig. 1(b), (d); right breast). The scale bars are 2 cm. The Figure 1(a), (b) images were obtained at TP0 – before NACT; the Figure 1(c), (d) images were obtained at TP1 – after one NACT cycle. Since three weeks separate the DCE-MRI acquisitions, the image slices cannot be perfectly registered. The image slice displayed for TP1 represents a best estimate for equivalence to the image slice for TP0. Enhancing tumor regions are clearly hyperintense in these $T_1$-weighted images. As examples, the red borders in Figure 1(a), (c), demarcate the radiologist-selected tumor ROIs in these image planes.

Figure 2 shows 12 zoomed pixel-by-pixel SSP parametric maps of the Figure 1 tumors, six from each subject. The upper six ((a)–(f)) are from the Figure 1(a), (c) non-pCR and the lower six ((g)–(l)) are from the Figure 1(b), (d) pCR case. The top row of maps ((a)–(c), (g)–(i)) for each patient was obtained at TP0 – before NACT initiation. The bottom row ((d)–(f), (j)–(l)) was obtained at TP1 – after one NACT cycle; three weeks. Besides the $\tau_i$ maps, the other pharmacokinetic parameters are $K_{\text{trans}}$ and $v_e$. The color scales ($K_{\text{trans}}$ in min$^{-1}$; $\tau_i$ in s) are given; $v_e$ is dimensionless.

We briefly divert to spatial- and population-averaged results. Figure 3 is a column graph showing the early therapeutic responses of whole tumor-averaged parameter values, further averaged over the pCR (black bars) and non-pCR (gray bars) sub-populations. The error bars reflect the considerable inter- and intratumor (Fig. 2) heterogeneity averaged. The parameters

![Image](https://example.com/image1.png)

**Figure 1.** Axial breast $T_1$-weighted images, obtained during CA bolus passage, from two representative subjects in the population. (a) The left breast, before therapy (TP0), of one of eight subjects deemed a non-pCR after 18 weeks of NACT. (c) The same subject after 3 weeks of NACT (TP1). (b), (d) Right breast images of one of three subjects declared pCR, obtained at TP0 and TP1, respectively. Each patient had grade 2 IDC breast cancer. The radiographic tumor outlines are marked in red in (a) and (c). The scale bars are 2 cm.
Figure 2. Zoomed shutter-speed DCE-MRI parametric maps of the two tumors shown in Figure 1. (a)–(f) Subject non-pCR; (g)–(l) pCR. (a)–(c), (g)–(i) were obtained pre-therapy (TP0), and (d)–(f), (j)–(l) 3 weeks into (TP1) the 18 week NACT course. The biomarkers $K_{trans}$, $K_{ep}$, $v_e$, $\tau_i$, and $\tau_c$ measure, respectively, CA extravasation kinetics, extracellular volume fraction, and mean intracellular water lifetime, and exhibit intratumor heterogeneity. Analyses of $\tau_i$ and $v_e$ relationships for 21 pairs of seven different ROIs within these maps (two outlined in yellow in (c)) indicate that the $\tau_i$ maps reflect metabolic activity: the smaller $\tau_i$, the greater the NKA turnover. Note the $\tau_i$ scale change.

Figure 3. Sub-population-averaged whole tumor biomarker values. The vertical axis measures the percentage change after the first three weeks of therapy (between time point TP0 and time point TP1). The bar colors represent the sub-populations after 18 weeks of therapy, discriminated by pathology analysis: black, pCR (n = 3); gray, non-pCR (n = 8). The RECIST parameter is a radiographic tumor size measure; the others are DCE-MRI kinetic parameters from tracer or SSP analyses: $K_{trans}$ (mainly CA extravasation), $k_{ep}$ (CA extravasation), and $\tau_c$ (mean intracellular water lifetime). The change in DCE-MRI biomarkers after three weeks, particularly those from SSP – including $\tau_c$ – are excellent predictors of therapeutic outcome after 18 weeks. For the responders, $\tau_c$ increases, signifying a therapeutically induced decrease in metabolic activity.

The vertical axis measures the percentage change in the biomarker after the first NACT cycle, i.e. in the three weeks between TP0 and TP1. The bar color (pCR versus non-pCR), however, is determined only after completion of the entire NACT therapeutic course (18 weeks), surgery, and pathology analyses. Though the mean tumor size (RECIST) decreased only slightly after three weeks, the DCE biomarkers showed larger changes. Interestingly, the SSP $K_{trans}$ and $k_{ep}$ parameters decreased for the pCR subpopulation, while $\tau_c$ increased. Overall, the ULR analysis found that the percentage changes in tumor mean $K_{trans}$ (tracer and SSP), $k_{ep}$ (tracer and SSP), and pixel $\tau_c$ histogram median after the first NACT cycle (at TP1 relative to TP0) were excellent discriminators of pCRs from non-pCRs, each with the ULR c statistic value of 1.0 (meaning complete separation), while the early RECIST percentage change was a poor predictor with c = 0.60. (The $v_e$ parameter has c values less than unity.) In addition, the absolute values of $K_{trans}$ tumor mean $K_{trans}$ (SSP) and $k_{ep}$ (tracer and SSP) were also effective (c = 1.0) early discriminators (not shown). After only one NACT cycle, changes in the tumor-averaged shutter-speed DCE biomarkers $K_{trans}$, $k_{ep}$, and $\tau_c$ are excellent predictors of the therapeutic outcome to be found after NACT completion. This is very encouraging. We have published a preliminary report with more results (40), and plan a full paper on these aspects. The tumor-averaged $K_{trans}$ (SSP) and $\tau_c$ values at TP3 significantly correlate with the RCB magnitude found by pathology analyses.

However, the significant intra- and intertumor heterogeneity (1–6) described above seriously calls for individualized tumor assessment (7). For the remainder of this paper, we return our focus to the intratumor parametric maps of the two representative Figure 1, 2 case studies. These allow in-depth deductive analyses of $\tau_i$ interpretation. In these, the subjects serve as their own controls. Figure 4 shows $\tau_i$ (ordinate), $K_{trans}$ (abscissa) scatter plots of the 228 tumor pixels from Figures 2(g), (i) (0.38 mL ROI) (a) and the 142 tumor pixels from 2(j), (l) (0.24 mL ROI) (b). These are for the representative pCR patient. The $\tau_i$ values in NMR spectroscopic cell suspension studies and in ROIs can be determined with precisions better than 5% (17) and 10% (30), respectively. However, single voxel DCE-MRI data have greater relative noise, which could diminish pixel $\tau_i$ precision. Figure 4(a), (b) appraises...
that in Figure 4(a). As shown in Figure 3, the large uncertainties occur for both small and large values for a very long duration (13,30,32). Interestingly, these greater depart the FXL for the FXR condition very extensively and/or not become very large for very long, and the system does not ever, that these occur only in regions where NMR Biomed. magnitude (the error bar is relatively independent of the mean). It indicates that the \( v \) precision is not particularly dependent on \( v \) magnitude (the error bar is relatively independent of the mean). It implies that the \( v \) accuracy is relatively independent of \( v \) precision. This is also found for ROI data (30).

The situation in Figure 4(b) (pCR, TP1; Figure 2(j)) is different from that in Figure 4(a). As shown in Figure 3, the large \( K_{\text{trans}} \) decrease is good news for the pCR patient. (The tumor image slice-averaged \( K_{\text{trans}} \) decreases from 0.19 min\(^{-1} \) at TP0 (Fig. 2(g)) to 0.04 min\(^{-1} \) at TP1 (Fig. 2(j)) – i.e. by 79%). However, the greatly diminished \( K_{\text{trans}} \) provides a difficult scenario for precise \( v \) determinations. The error bars reflect this. Nonetheless, one can discern that, on the whole, \( v \) values are larger than in Figure 4(a). (The tumor image slice pixel-averaged \( v \) goes from 0.32 s at TP0 (Fig. 4(a)) to 0.39 s at TP1 (Fig. 4(b)) – a 22% increase.) This result reinforces the notion that the \( v \) magnitude itself does not decrease with \( K_{\text{trans}} \), mostly, the precision of its determination becomes poorer. When clinically indicated, this precision can be considerably improved by moving up to the approved triple CA dose.

A 2D scatter plot is an effective way to take advantage of two responsive biomarkers (52). The slight negative spatial \( v \) \( K_{\text{trans}} \) correlation apparent to the eye in the Figure 2(g), (l) parametric maps can also be barely discerned in Figure 4(a). The Pearson correlation coefficient is –0.23. The substantial decrease in \( K_{\text{trans}} \) values after one NACT cycle (TP0, (a), TP1, (b)) is quite obvious. It is also clear from Figure 4(b) that the \( v \) and \( K_{\text{trans}} \) magnitudes are independent: they are not numerically correlated by the analysis. As we have seen, the pCR tumor- and population-averaged \( v \) increases after therapy (Fig. 3). From Figures 2(i), (l) and 4(a), (b), we can detect an overall increase in \( v \) after NACT. (The whole tumor-averaged \( v \) increases from 0.27 s to 0.41 s – i.e. by 52%.) Figure 2(l) suggests that this is localized mainly in the tumor core. From the \( v \) perspective, we can see from Figure 3 (based on whole tumor averages) that the result for the patient of Figure 2(l), (l) represents the most conservative of the three pCR cases. Importantly, there are no significant \( v \) differences between core and rim after therapy (Fig. 2(k)). As we will see, the tumor core \( v \) increase by therapy is due to a \( P_W \) decrease.

**Parameter heterogeneity, relationships, and therapy responses**

Though the tumors appear relatively homogeneous in Figure 1, the SSP parametric maps exhibit significant intratumor heterogeneity that appears anatomic in nature. For example, the TP0 \( K_{\text{trans}} \) maps of each tumor display elevated values in the tumor rim relative to the core. In Figure 2(a), (g), the \( K_{\text{trans}} \) variation exceeds a factor of 10. This pattern is common. It is observed in malignant human tumors – breast (37,46), osteosarcoma (35), head and neck (43), and soft tissue sarcoma (53), in spontaneous murine breast tumors (39), and in implanted rodent cerebral gliosarcoma (32), RIF-1 (33) and prostate (38) tumors. However, it is by no means universal: tumors with bright \( K_{\text{trans}} \) cores (or multiple cores) have been reported for malignant human breast (18,34,35,41,42), head and neck (44), and prostate (47) tumors, and in implanted rat glioma (54). The observation of this diversity is very promising for individualized imaging.

Furthermore, there are often spatial correlations between the imaging biomarkers. In the Figure 2 \( K_{\text{trans}} / v \) pairs (especially (a)/(c) and (j)/(l), less so for (d)/(f) and (g)/(i)), regions with relatively elevated \( K_{\text{trans}} \) values generally have relatively smaller \( v \) values, and vice versa. (The non-pCR core \( v \) is quite large, 1 s (Fig. 2(c),l)). This is also often observed in other human malignant tumors – breast (34,35,37), osteosarcoma (35), head and neck (43,44), and prostate (36) – and in spontaneous murine breast tumors (39). However, this negative correlation is not always the case: there are counter-examples in the human breast (41,42) and in implanted rodent gliosarcoma (32,55), RIF-1 (33), and prostate (38) tumors. Thus, the sign of the biomarker spatial correlation is independent of the intratumor heterogeneity spatial pattern.
changes reflect membrane permeability changes

For globular cells, Equation [3] gives \( \tau_i^{-1} = 6p_W d^3 \); \( p_W \) is the membrane water permeability, \( d \) the cell diameter. For a spherical cell with a conservatively large \( d \) value (15 \( \mu \)m (6)), \( \tau_i^{-1} = 4000p_W (\tau_i \text{ in } s, \ p_W \text{ in cm } s^{-1}) \). For a spherical cell with a typical \( p_W \) value (1.4 \times 10^{-8} \text{ cm s}^{-1} (10)), \( \tau_i^{-1} = 8.4/d \) (in \( \mu \)m). Thus, \( \tau_i^{-1} (k_0) \) is linearly related to \( p_W \) and linearly related to \( d^{-1} \), with different coefficients. Do observed \( \tau_i \) variations reflect changes in \( p_W \), in \( d \), or in both? In this paper, we compare relative (\%) changes in \( \tau_i^{-1} \) values within human breast tumors with the accompanying \( d^{-1} \) changes, to show that \( p_W \) dominates \( \tau_i \).

Let us inspect a \( \tau_i \) variation in Figure 2. Consider the \( \tau_i \) map of the non-pCR patient at TP0 (Fig. 2(c)). We choose an ROI representative of the annular tumor rim: we designate it RN0, for representative of the annular tumor rim (Fig. 2(c)). RN0 comprises 55 pixels (66 mm²; 9.4 \times 10^5 pixels). Thus, \( \tau_i (k_0) \) is dominated by a \( d^{-1} \) term, while \( k_0 \) is not very large, even for the non-pCR individual (Fig. 2(c), (f)). Thus, there is a negative \( \tau_i \) \( K^{trans} \) correlation in (therapy) time as well in space. Importantly, the \( \tau_i \) increase and \( K^{trans} \) decrease after 3 weeks of NACT predict very well that no RCB will be surgically found after 15 more weeks of therapy. This response is representative of the pCR tumor- and population-averaged results, and occurs usually before significant tumor size decrease (Fig. 3). This is also true for the \( K^{trans} \) decrease caused by therapy on soft tissue sarcoma (53).

We also see \( \tau_i \) increase/\( K^{trans} \) decrease after a different therapy on a spontaneous murine breast tumor (39). It is important to note that the rather large tumor \( \tau_i \) value (0.56 s, image slice average) in Figure 2(i) is obtained while \( \tau_i \) is also very large, 0.87 image slice average, Figure 2(k). If \( \tau_i \) is large, then \( \tau_i^{-1} = (1 – \tau_i) \) is small. This result reinforces the fact that, contrary to what we might expect, \( \tau_i \) magnitude does not decrease with \( \tau_i \). This is because of its intensive nature.

Changes in \( \tau_i \) reflect membrane permeability changes
DISCUSSION

As described above, the analysis of DCE-MRI time-course data with any formulation of the tracer pharmacokinetic paradigm (most common by far) is incorrect. It neglects the finite kinetics of the inter-compartmental water exchange equilibria that preceed the rate limiting step of CA extravasation. This causes systematic changes in the DCE-MRI pharmacokinetic parameters, $k^{\text{trans}}$ and $v_w$. In particular, spatially averaged $k^{\text{trans}}$ is disproportionately depressed in malignant tumors of the breast (13,18,34,35,46) and prostate (30,47). The SSP allows extremely high specificity in the detection of these cancers: specificity not possible with tracer analysis. This makes effective cancer detection (13,18,34,46,47) and therapy prediction (40,53) practical.

Here, however, we focus on the second benefit of the SSP, access to the water exchange kinetics themselves. This is not possible with the tracer paradigm, where water is not considered molecular but merely a continuum filling tissue compartmental spaces. The equilibrium trans-cytollemmal water exchange kinetics are measured by $\tau_i$, the reciprocal of the unidirectional rate constant for water efflux, $k_{io}$.

Changes in $\tau_i$ reflect changes in the driving membrane P-type ATPase ion pump turnover

The results above indicate that the intratumor $\tau_i$ variations observed in human breast cancer are dominated by $P_W$ differences. If they were dominated by $d$, the $\tau_i^{-1} (k_{io})$ percentage increases and $d$ percentage decreases would be similar. Still, are the differences observed in $P_W$(passive), $P_W$(active), or in both?

Almost all cells have a driving membrane P-type ATPase ion pump enzyme, which serves to generate transmural ion gradients and membrane potentials (26). For yeast cells, this is the H+-ATPase, Pma1 (17,26). For mammalian cells, it is NKA (26). The forward reaction catalyzed by NKA can be written as in Equation [5], where intracellular adenosine triphosphate (ATPi) is hydrolyzed to ADPi and Pi,

$$\text{ATPi} + 2 \text{K}_i + 3 \text{Na}_o \rightarrow \text{ADPi} + \text{P}_i + 2 \text{K}_o + 3 \text{Na}_i \quad [5]$$

extracellular potassium ($K_o$) is transported into the cell, and intra-cellular sodium ($Na_i$) is expelled. Table 1 summarizes the literature on the dependence of equilibrium transcytlolemmal water exchange kinetics ($\tau_i^{-1}$) on driving membrane P-type ATPase ion pump gene dosage, substrate concentration, and specific inhibitor concentration in yeast suspension, perfused rat heart,

| Table 1. $\tau_i^{-1} (k_{io})$ reflects turnover of driving membrane P-type ATPase ion pump |
|---------------------------------------------------------------|
| P-type ATPase ion pump | Yeast$^{17}$ | Cardiomyocyte$^{28}$ | Erythrocyte$^{61}$ |
| Gene dosage | ↑↑ | ↓↓ | ↑↑ |
| Substrate | ATP, $K_o$ | ↑↑ | → | ↑↑ |
| Specific inhibitor | Ebselen, Ouabain | ↑↓ | → | ↑↓ |

17 Measured (17); 28 measured (28); 61 inferred (61).
and erythrocyte suspension studies. Without exception, \( \tau_i^{-1} \) increases with gene copy number and substrate concentration, and decreases with extracellular inhibitor concentration. (Ebselen and ouabain are specific inhibitors of Pma1 (17) and NKA (26), respectively.) This is strong evidence that \( \tau_i^{-1} (k_{mi}) \) is dominated by \( P_{W,\text{active}} \), which in turn is driven by P-type ATPase ion pump turnover. The greater the turnover, the faster the exchange. All of these model systems were homoeostatic for the Table 1 entries.

Figure 6 presents a schematic diagram of the general molecular mechanism we have proposed (17) for active trans-membrane water cycling. The passive water exchange (\( P_{W,\text{passive}} \)) equilibrium is indicated at the top right. It involves simple water diffusion through the phospholipid bilayer, transport through aquaporin channels (60,61), and leakage through membrane protein transporters (25). Active trans-membrane water cycling (\( P_{W,\text{active}} \)), which can have three times the \( P_{W,\text{passive}} \) flux (17), almost certainly involves water co-transporting membrane symporters (24). In the diagram, active water efflux is pictured as passing through NKA, and influx through the sodium glucose co-transporter (SGLT (62)), but this is only for the purpose of illustration. It is not yet known which symporter molecules dominate active water cycling: there are a number of candidates, SGLT certainly being one of them (24). By themselves, aquaporins catalyze only passive transmural water transport. However, to the extent to which they co-localize with substrate transporters, say K+ channels (60) or NKA (63), they may participate in active trans-membrane water cycling.

Because it maintains the trans-membrane ion gradients that drive much secondary active transport and produce the membrane potential, one can argue that NKA is the most important enzyme in mammalian biology. Because of the particular, dual (‘vectorial’ and ‘scalar’ (64)) characteristics of the reaction catalyzed by NKA (Equation [5]), measurements of its activity have always been adapted to the nature of the sample. For solubilized, purified enzyme or tissue homogenate preparations, one cannot measure the kinetics of (vectorial) ion transport. Thus, spectrophotometric (65) or radiolabeled (\( ^{32}\text{P} \)) assays (66) of the rate of ATP hydrolysis are used. On the other hand, for intact cells in culture or in tissue preparations, one cannot easily measure the kinetics of the (scalar) intracellular ATP hydrolysis caused by NKA activity. However for such samples, voltage clamp current, ion-selective (Na+/K+) microelectrode response, radioisotope (\( ^{22}\text{Na} / ^{38}\text{Na} / ^{85}\text{K} / ^{85}\text{Rb} \)) uptake/release (67–70), or \( ^{22}\text{Na}, ^{85}\text{Rb} \) MR spectroscopic (71,72) methods can be used to measure NKA-driven trans-membrane ion transport kinetics. This is how it was learned that, when the concentrations of the other reactants and products have typical values, the intracellular Na+ concentration, [Na+], is generally the rate-determining factor (71,73,74). It is also possible to measure [Na+] using a fluorescent indicator (75). A breakthrough found that phospholipid vesicles reconstituted with purified NKA facilitated measurement of both ATP hydrolysis and ion transport (66). This allowed confirmation of the NKA reaction stoichiometry (66).

It is obvious that each of these methods is best suited to macroscopically homogeneous samples. None are particularly appropriate for use with normally heterogeneous tissue. (NKA distributions can be mapped histologically (76).) Except for the microelectrode approaches, these do not involve spatial encoding; and one cannot insert electrodes in all of the cells of a tissue. Furthermore, many of these methods directly measure only net NKA activity, not homeostatic NKA turnover. (An analogous problem arises measuring net versus steady-state water transport kinetics (15).) A \( ^{24}\text{Na} \) study reveals the existence of an equilibrium transmural Na+ exchange process in the cardiomyocyte over an order of magnitude faster than net Na+ transport (69). However, the radioisotope approach has been generally abandoned for ~20 years, and deemed too problematic for even tissue preparations (73). As far as we are aware, the NKA turnover has never been measured, let alone mapped, in a living animal or human subject. Therefore, \( \tau_i^{-1} \) measurement offers the possibility of quantifying perhaps the most crucial ongoing cellular metabolic turnover. For a spherical cell with \( d = 12 \, \mu \text{m} \), \( \tau_i = 400 \, \text{ms} \) signifies active cycling of \( 7 \times 10^{13} \)
H2O molecules s⁻¹/cell (17). If the stoichiometric flux ratio \( \text{flux}_{\text{Na}}^{\text{trans}} = (10^{-3} \text{ to } 10^{-2}) \text{flux}_{\text{H2O}} \) (24) pertains, the Na⁺ flux is \( 10^{-1} \text{ to } 10^{-2} \) Na⁺ ions s⁻¹/cell. If \( r_i \) maps are reciprocal NKA turnover maps, they represent high-resolution metabolic images.

We show that \( r_i \) exhibits significant intratumor heterogeneity in human breast cancer in vivo. That the biomarker spatial correlations are never perfect and that counter-examples exist suggest that, when seen, these are not numerical co-variance artifacts resulting from the three parameter fittings of DCE-MRI data time-courses. They appear to be physiological correlations.

The synergism of two responsive imaging biomarkers can be very powerful. In this case, one reports on kinetic processes occurring outside cells: \( \tau_{\text{trans}} \) measures mostly the microvascular CA extravasation/tissue arrival rate. The other (\( r_i \)) measures metabolic fluxes inside cells. Since CA employs a paratothelial cellular pathway (77,78), it serves also as a surrogate for paracellular extravasation of plasma solutes with similar molecular sizes. Nutrients, particularly glucose (79), represent a crucial sub-class of these. Though glucose has specific transcellular transporters, when \( \tau_{\text{trans}} \) is relatively large it is likely that additional paracellular glucose extravasation is also relatively large. When \( \tau_{\text{trans}} \) is relatively large but \( r_i \) is relatively small, as in the rims of both Figure 2 tumors before therapy, it may be signaling that greater nutrient delivery enables faster cell metabolism. The pre-therapy biopsy core for non-pCR showed clearly that the parametric rim seen in Figure 2(a)–(c) corresponds to a band with the greatest density of invasive carcinoma cells and inflammatory lymphocytes. Figure 2 suggests that NACT on the pCR tumor causes rim tissue to move from larger \( \tau_{\text{trans}} \)/smaller \( r_i \) to smaller \( \tau_{\text{trans}} \)/smaller \( r_i \) and core tissue from smaller \( \tau_{\text{trans}} \)/smaller \( r_i \) to smaller \( \tau_{\text{trans}} \)/larger \( r_i \). We find very similar behavior with a different therapy (phosphatase 2A re-activation) on a spontaneous murine breast tumor (39). Perhaps this pathway is common for therapy-induced tumor regression. The most parsimonious explanation is that a \( \tau_{\text{trans}} \) decrease echoes a nutrient delivery decrease and then, subsequently, there is a decrease in metabolic activity signaled as a \( r_i \) increase.

The correlation of the results we see at TP1 with the pathology results after 15 additional weeks of therapy (Fig. 3) is very encouraging. This is crucial for early, personalized therapy evaluation and adjustment. The two cases reported here exemplify this. If we had sufficient statistical experience that our approach informed clinical decisions, the therapy of the non-pCR patient might have been altered after TP1, and the therapy of the pCR patient might have been ended after TP3, or not switched after TP3.

The region of positive \( r_i \), \( \tau_{\text{trans}} \) correlation in the rat gliosarcoma rim (32,55) coincides with the region of the implanted rat glioma that stains positive with EFS (55,80), a marker for hypoxia. This correlation is also positive for the RIF-1 tumor (33), which is known to be highly hypoxic (81). New results in a rat model of head and neck cancer show extensive overlap of an elevated \( r_i \) region with that of EFS staining (55). Also, increased tumor \( r_i \) strongly correlates with survival times of human head and neck cancer patients (H. Poptani, personal communication). When \( r_i \) is relatively large, the NKA activity is relatively small. Thus, it makes sense that \( \tau_{\text{trans}} \) and \( r_i \) are large and positively correlated in tissues where the cells may have entered a hypoxic state. Though the delivery of glucose is sufficient, it is not metabolized efficiently w.r.t. ATP synthesis (81,82).

The \( \tau_{\text{trans}} \) value can be sensitive to the degree of tissue vascularization. If vascularization is low, the apparent \( \tau_{\text{trans}} \) value decreases because, after extravasation, CA arrival in the tissue also requires diffusion (83). We do observe very small \( \tau_{\text{trans}} \) and relatively large \( r_i \) in breast adipose tissue (34,35). Presumably, this is due to the low vascularization. Also, in necrotic areas, one would expect \( \tau_{\text{trans}} \) to be relatively small and \( r_i \) to be relatively large, since cell metabolism should be slow.

Certainly, the spatial resolution of \( \text{H_2O} \) MRI does not compare with that of the optical microscopy of pathology. A typical high-resolution MRI pixel covers \( 4 \times 10^4 \) high power ‘20×’ pixels (0.5 μm)² from a modern digital pathology microscope (84). However, the results presented here introduce the potentially highest resolution in vivo metabolic imaging.

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APPENDIX A: CHANGES IN $\tau_i$ REFLECT MEMBRANE PERMEABILITY CHANGES

Analyses of non-pCR subject pre-therapy RN0 and CN0 ROI (Fig. 2(b), (c)) parametric relationships

Consider the $\tau_i$ map of the non-pCR patient at TP0 (Fig. 2(c)). We choose an ROI representative of the annular tumor rim: we designate it RN0 for rim, non-pCR, at TP0. The $\tau_i$ value for RN0 is 0.60 s for a conservative ROI representative of the outer core, CN0, the $\tau_i$ value is 0.81 s (the inner core has even larger $\tau_i$ values). These ROIs are outlined with yellow borders in Figure 2(c). The ratio $\left(\frac{\tau_i[R]}{\tau_i(C)}\right)^{-1}$ is 1.7/1.2 = 1.4: there is a 40% increase in $\rho_0$ for the rim over the core. Thus, the relationship $\frac{P_{RN0}/P_{CN0}}{(d_C^{-1} - d_R^{-1})} = 1.4$ must be satisfied. Now, consider the accompanying $v_e$ map (Fig. 2(b)). By definition, $(1 - v_e) = v_e$ where $v_e$ is the intracellular volume fraction. Furthermore, $v_e = (n/V_r) v$, where $(n/V_r)$ is the number of cells in the ROI (or voxel) volume ($V_r$) and $V$ is the mean individual cell volume (Equation [1]). The quantity $(n/V_r)$ is the ROI (or voxel) mean cell number density ($n$): a few hundred thousand cells/voxel. Thus, $(1 - v_e) = v_e = V_r/V$. For spherical cells, the mean effective cell diameter $d = V^{1/3} = (1 - v_e) / v_e$. For an ROI pair labeled R and C, the ratio $\frac{(1 - v_{e[R]})}{(1 - v_{e[C]})}$ is 0.38 and 0.73, respectively. For these values, the ratio $(d_{R}^{-1} - d_{C}^{-1})/p_C/p_R$ is 0.76 for the Figure 2(b) RN0 and CN0 ROIs (those marked in Figure 2(c)), the $v_{e[R]}$ and $v_{e[C]}$ values are 0.38 and 0.73, respectively. The relationship $(d_C^{-1} - d_R^{-1})/p_C/p_R = 0.76$ must be satisfied.

The red curve in Figure A1 is the trace of all points, in a 3D space of tissue cellular properties, which simultaneously satisfy the experimental $P_{RN0}/P_{CN0}((d_C^{-1} - d_R^{-1})/1.4) = 1.4$ and $(d_C^{-1} - d_R^{-1})/p_C/p_R = 0.76$ relationships for the rim and core ROIs of Figure 2(c), (b), respectively (taking $d_C = d_R = d_0$). The axes are $P_{RN0}/P_{CN0}$ (vertical), $(d_C^{-1} - d_R^{-1})$ (inverse cell diameter ratio = $(d_C^{-1} / d_R^{-1})$), and $p_C/p_R$ (cell density ratio). This is very informative. A black circle marks the point on the red curve where $p_C/p_R = 0.6$, the value determined from histology analysis of the biopsy core (see text). Its other coordinates are $d_0^{-1} = 0.9$, and $P_{RN0}/P_{CN0} = 1.6$. If anything, the permeability ratio is larger than $[(c_{R}[R])^{-1} / c_{R}[C]]^{-1} = 1.4$. To allow for the $\rho$ determination, a conservatively generous range for $p_C/p_R$ of 0.9 to 0.3 is shaded gray in the Figure A1 horizontal plane. The dot-
dash red projection shows that the allowed \((d_{\text{d}})^{-1}/(d_{\text{c}})^{-1}\) values vary from 0.8 to 1.1 in the gray shaded region. This indicates that, averaged over the many different cell types and sizes of these ROIs (RN_{0} 39 million cells; CN_{0} 19 million cells), the mean cell diameter in the rim is approximately the same as in the core. The \(P_{W}[R]/P_{W}[C]\) values (vertical) over the gray shaded area range from 1.8 to 1.3 (80–30% increases in rim \(P_{W}\), relative to core). We conclude that \(P_{W}\) is substantially larger in the rim of the untreated non-pCR tumor than in the core: \(r_{i}\) variation is not due to \(d\) variation.

**Other Figure 2 ROIs**

Besides RN_{0} and CN_{0} (Fig. 2(b), (c)), we have chosen five other representative ROIs: RN_{1} and CN_{1} (Fig. 2(e), (f)), RC_{0} and CC_{0} (Fig. 2(h), (i)), and CC_{1} (Fig. 2(k), (l)) (in the ROI labels, the first character is R (rim) or C (core), the second character is N (non-pCR) or C (pCR), and the subscript is 0 (TP_{0}) or 1 (TP_{1})). Taken two at a time, these seven ROIs afford 21 (=7!/(5!2!)) ROI pairs and thus two \(r_{i}\), and (1 – \(v_{0}\))/\(v_{0}\) ratios. We take these ratios such that the larger \(r_{i}\) (\(k_{0}\)) value (smaller \(r_{i}\) \(k_{0}\)) is the numerator: e.g., \((r_{1}[S])^{-1}/(r_{1}[L])^{-1}\) > 1. For the 21 ratios, \((r_{1}[S])^{-1}/(r_{1}[L])^{-1}\) ranges from 1.05 to 3.58, and averages 1.74, a 74% increase. The companion \(((1 – v_{0}[S])/(1 – v_{0}[L]))^{-1/3}\) ratios \(=((d_{1}[S])^{-1}/(d_{1}[L])^{-1})|\(r_{0}/r_{0}\)) range from 0.67 to 1.50, and average 1.05, a 5% increase that is not statistically different from 0%. The details are given in Table A1. The inter-ROI pair SDs are also given. Since these represent inter-subject and/or inter-session comparisons, their relatively small SD sizes are quite encouraging for the pseudo-absolute nature of these imaging biomarkers.

**Literature results**

Table A1 also summarizes literature reports of experimentally induced \(r_{i}\) changes that also have accompanying measurements allowing calculation or estimation of \(d\) changes. These span

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**Table A1.** Trans-membrane water exchange increases are disproportionately larger than cell size decreases

| System                        | AML cells (n = 5) | Yeast cells (n = 6) | Murine myocardium in vivo/ex vivo (n = 17) | Human breast tumors in vivo (21 ROI pairs) |
|-------------------------------|------------------|--------------------|-------------------------------------------|------------------------------------------|
| Smaller \(r_{i}\) (s^{-1})    |                  |                    |                                           |                                          |
| \(k_{0}\) (larger \(r_{i}\), \(r_{i}[L]\)) | 1.4 (±43%) \(^d\) | 1.5 (±6%) \(^b\)  | 2.3 (±29%) \(^c\)                         |                                          |
| Larger \(r_{i}\) (s^{-1})    |                  |                    |                                           |                                          |
| \(k_{0}\) (smaller \(r_{i}\), \(r_{i}[S]\)) | 6.8 (±57%) \(^d\) | 3.1 (±3%) \(^b\)  | 5.3 (±43%) \(^f\)                         |                                          |
| \(r_{i}\) (\(k_{0}\)) change |                  |                    |                                           |                                          |
| \((d_{1})^{-1}\) for smaller \(r_{i}\) | 1.0 (±10%) \(^g\) | 2.2 (±2%) \(^b\)  | 0.038 (±6%) \(^{c,h}\) (\(\mu m^{-1}\)) |                                          |
| \((d_{1})^{-1}\) for larger \(r_{i}\) | 1.1 (±4%) \(^g\) | 2.4 (±2%) \(^b\)  | 0.051 (±5%) \(^{c,h}\) (\(\mu m^{-1}\)) |                                          |
| Change                        | 10% increase     | 9% increase        | 34% increase                              | 5% increase (±22%) \(^{c}\) this work |

\(^{a}\)Control cells.
\(^{b}\)N\(_{2}\) gasification.
\(^{c}\)L-NAME-treated mice, in vivo.
\(^{d}\)Cisplatin-treated cells.
\(^{e}\)O\(_{2}\) gasification.
\(^{f}\)Control mice, in vivo.
\(^{g}\)\(d' = (p_{i})^{1/3}\).
\(^{h}\)Cylindrical diameter by microscopy on ex vivo fixed tissue.
\(^{i}\)Average for 21 ROI pairs in Figure 2 (see text).
\(^{j}\)Average \(((d_{1})^{-1}/(d_{1})^{-1})|\(r_{0}/r_{0}\)| for 21 ROI pairs in Figure 2 (\(p_{i}\) is cell number density); not statistically different from 0%.
three model systems: two cell suspensions (17,31) and murine myocardium (58). In the two cell suspension studies, the yeast (17) and AML (31) cell densities were not radically changed by O₂ gasification and cisplatin treatment, respectively. Since the cells are spherical, the ratio of $p_i^{1/3}$ (where $p_i$ is the measured intracellular water mole fraction, 'population') values approximates the $(v_i)^{-1/3}$ ratio and thus the $(d_i')^{-1}$ ratio. Table A1 shows that the 9% and 10% $(d_i')^{-1}$ increases caused by O₂ gasification of the yeast cells (17) and cisplatin treatment of the AML cells (31), respectively, are much smaller than the concomitant 110% and 390% $\tau_i^{-1}$ increases. In the mouse heart, in vivo $\tau_i^{-1}$ was observed to decrease after seven weeks treatment with an NO biosynthesis inhibitor, $\text{Nω}$-nitro-L-arginine methyl ester (L-NAME) (58). The ex vivo fixed tissue (cylindrical) cell $d$ values were microscopically determined for control and L-NAME treated mice. Systematic $d$ errors from tissue fixing must be hard to avoid. As seen in Table A1, the 130% $\tau_i^{-1}$ increase from L-NAME treated to control is accompanied by only a 34% increase in $d^{-1}$. The AML study (31) also included microscopy before and after the perturbation. The consistent nature of these model system results and our in vivo human breast tumor findings makes a compelling case that $\tau_i^{-1}$ changes are dominated by $P_W$ changes.