Computational fluid dynamics with imaging of cleared tissue and in vivo perfusion predicts drug uptake and treatment responses in tumors

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Abstract

Understanding the uptake of a drug by diseased tissue, and the drug’s subsequent spatial-temporal distribution, are central factors in the development of effective targeted therapies. However, the interaction between the pathophysiology of diseased tissue and individual therapeutic agents can be complex, and can vary across tissue types and across subjects. Here, we show that the combination of mathematical modelling, of high-resolution optical imaging of intact and optically cleared tumour tissue from animal models, and of in vivo imaging of vascular perfusion predicts the heterogeneous uptake, by large tissue samples, of specific therapeutic agents, as well as their spatiotemporal distribution. In particular, by using murine models of colorectal cancer and glioma, we report and validate predictions of steady-state blood flow and intravascular and interstitial fluid pressure in tumours, of the spatially heterogeneous uptake of chelated gadolinium by tumours, and of the effect of a vascular disrupting agent on tumour vasculature.
Introduction

Mathematical modelling of biological tissue is increasingly used to better understand complex biological phenomena, such as the development of disease. This developing paradigm of computational experimentation can enable subtle interventions to be performed in a manner that would be challenging or impossible in a conventional experimental setting. In this study we present a framework for performing realistic computational experiments that naturally incorporates the variability and heterogeneity found between biological samples. It allows large tissue samples to be imaged and treated as living specimens, by combining cutting-edge optical and in vivo imaging techniques with mathematical modelling. We have named our framework REANIMATE (REAlistic Numerical Image-based Modelling of biologicAl Tissue substratEs) (see Figure 1 for an overview diagram).

Optical imaging of cleared tissue can provide three-dimensional data detailing complex, interacting structures (such as blood vessel networks, cell nuclei, etc.), which can be explored, across entire organs, and at resolutions of a few microns, by using fluorescently-labelled probes that bind to specific structures. Our use of large, high resolution structural images in computational simulations, rather than relying on small, isolated samples or synthetically-generated substrates, is a key development, and has required the development of new image analysis and computational modelling approaches. Furthermore, by incorporating in vivo imaging (in particular, magnetic resonance imaging (MRI)), REANIMATE can incorporate quantitative measurements.

Capturing the physiological variation in complete tissue specimens is particularly useful in tumors, which can be highly heterogeneous, both between tumor types, tumor deposits and even within individual tumors. This results in substantial differences in, for example, drug delivery, oxygenation and gene expression, with associated differences in therapeutic response and resistance. Effective therapy normally requires drugs to be delivered to the site of disease, at as high a concentration as possible, but avoiding significant toxicity effects in healthy tissues, whilst sub-optimal exposure can limit treatment efficacy, induce exposure-mediated resistance mechanisms, or even stimulate tumor growth.

This complex physiological-pharmacological landscape requires careful analysis in order to be fully understood. The numerical modelling component of REANIMATE consists of two steps: first, a solution is sought from a set of coupled fluid dynamics models that describe...
steady-state vascular and interstitial fluid transport; second, the steady-state solution (or set of solutions) is used to parameterise a time-dependent model that describes the vascular and interstitial uptake of exogenously administered material. This can be used, for example, to model the heterogenous pharmacokinetics of drug or imaging contrast agents, or delivery of individual particles (e.g. T-cells, antibodies), and terms can be introduced to describe drug targeting and metabolism.

For the predictions made by our, or any, computational experiments to be confidently accepted, careful experimental validation must be performed. As a first evaluation, we have used REANIMATE to: 1) study the spatially heterogeneous uptake of a gadolinium-based MRI compound (which allowed us to compare numerical modelling solutions with ground-truth \textit{in vivo} data); and 2) investigate the effect of the vascular disrupting agent (VDA) Oxi4503 on tumor vasculature. These results provided a rich, three-dimensional framework for probing spatially heterogeneous tumor drug delivery and treatment response.

\textbf{Results}

\textit{Preparation of tissue substrates for mathematical modelling}

We began the development of the REANIMATE framework by studying SW1222 and LS174T human colorectal carcinoma tumors, implanted subcutaneously on the flank of immunocompromised mice. These tumor types have been extensively studied, by our group and others, with SW1222 tumors displaying greater cell differentiation, more uniform vasculature and greater perfusion than LS174T tumors.\textsuperscript{9-15} Tumors of each type (n=5 of each) were grown subcutaneously in mice for 10 to 14 days, and then administered fluorescently-labelled lectin (AlexaFluor-647) via a tail vein, in order to fluorescently label vascular structures in the tumors.\textsuperscript{2} Following a circulation time of 5 minutes, tumors were resected, optically cleared with benzyl-alcohol / benzyl-benzoate (BABB), and imaged, intact, with optical projection tomography (OPT).\textsuperscript{16}

Depending on the size of the tumor, our OPT images exhibited a variable background autofluorescence signal, with a decrease in signal intensity towards the centre due to less effective optical clearing. This was corrected by subtracting a three-dimensional Gaussian-filtered copy of the data, to normalise variations in signal intensity. Blood vessels were then segmented from OPT images using Frangi filtering\textsuperscript{17} and thresholding, and converted into graph format with a skeletonisation algorithm. These spatial graphs consisted of nodes
(branch points) and vessel segments, and were typically composed of 30,000 to 200,000 nodes. Examples of segmented, whole-tumor blood vessel networks from example LS174T and SW1222 tumors are shown in Figure 2.

We compared vessel architecture from SW1222 and LS174T tumors against previously published data (obtained using a range of imaging techniques, and principally derived from our own published studies), which showed that vessel architecture was preserved during tissue clearing, and that our image processing algorithms accurately reproduced vessel networks (see sections 1.2 and 1.2 in the Supplementary Information).

**REANIMATE steady-state simulation in subcutaneous colorectal carcinoma xenografts**

Our next aim was to use whole-tumor blood vessel networks as the substrate for simulations of steady-state fluid dynamics. Our mathematical model comprised of coupled intravascular and interstitial compartments, with exchange mediated by vascular permeability and described by Starling’s Law. Blood flow and interstitial delivery were modelled using Poiseuille flow and Darcy’s law, respectively, and the model was optimised over the entire tumor through the prescription of the pressure boundary conditions at peritumoral boundary vessels. We performed our initial simulations on a set of LS174T and SW1222 colorectal adenocarcinoma xenografts.

As shown in the summary of simulated parameter values in Supplementary Table 1, solutions to our mathematical model predicted significant differences between SW1222 and LS174T tumors, in blood flow, blood velocity and vessel wall shear stress, which are consistent with their known characteristics. Example spatial distributions of each of these REANIMATE parameters are shown in Figure 3, in which vascular parameters (blood flow and pressure) are displayed as colored vessel segments and interstitial parameters (interstitial fluid pressure (IFP), interstitial fluid velocity (IFV) and perfusion) as overlaid color fields. Results are shown for example LS174T (Figure 3a-e) and SW1222 tumors (Figure 3f-j).

Key to the interpretation of these results was our ability to compare them directly with equivalent in vivo imaging data (in this case arterial spin labelling magnetic resonance imaging (ASL-MRI)), which can be used to quantify perfusion, noninvasively. Perfusion is a measure of the rate of delivery of fluid to biological tissue, and is dependent on blood flow, vascular permeability and interstitial density, amongst other factors. Comparisons of ASL-MRI in vivo measurements and REANIMATE predictions are shown in Figures 3c (LS174T) and 3h (SW1222), which shows a clear correspondence between the two data types.
Statistical analysis revealed no significant differences between predicted and measured perfusion values (p<0.01, Kolmogorov-Smirnov). Scatter plots of both measurements are shown in Supplementary Figures 1a and b, which revealed a significant correlation between the two measurements (LS174T, $r^2 = 0.82$, p<0.001; SW1222 $r^2 = 0.89$, p<0.001; Pearson test). Likewise, in both in vivo measurements and simulations, perfusion was distributed heterogeneously throughout the tumors, with markedly raised values at the periphery of both types of colorectal tumor. This spatial distribution is characteristic of solid tumors, particularly subcutaneous xenograft models.\(^{19}\) However, some regional differences were also evident between simulated and ASL perfusion values, but which could have been caused by errors in either value, and/or errors in the spatial registration of the two types of data.

Both in vivo measurements and simulations showed that SW1222 tumors were better perfused than LS174T tumors, which is again consistent with the results of previous studies.\(^{20}\) However, perfusion at the centre of SW1222 tumors was much greater than in LS174T tumors, both in simulations and in vivo data. On average, we found that simulated perfusion values in LS174T tumors matched those measured in vivo with MRI (0.18 ± 0.07 and 0.19 ± 0.08 mL g\(^{-1}\) min\(^{-1}\)) for simulated and in vivo measurements, respectively), whilst SW1222 estimates were slightly larger, but of the same order as in vivo measurements (0.33 ± 0.18 and 0.73 ± 0.03 mL g\(^{-1}\) min\(^{-1}\)).

REANIMATE also predicted elevated interstitial fluid pressure in both tumor types, with typical values in the range 12 to 25 mm Hg in the center of tumors, and which declined towards the periphery. This, again, is consistent with the known characteristics of these types of tumor, and no significant difference was measured between LS174T and SW1222 tumors.\(^{21}\) However, in both tumor types, IFP was not uniformly distributed, but instead varied by up to 10 mm Hg within the center of individual tumors. IFP was raised at the location of perfused vasculature, producing intratumoral advection effects (as can be seen in the interstitial velocity images in Figures 3e and 3j). These results agree with previous measurements of IFP and IFV from our own laboratory, in which mean IFP was measured to be 16 ± 5 mm Hg in LS174T tumors and 13 ± 2 in SW1222 tumors, for a tumor volume of 0.1 cm\(^3\).\(^{21}\)

As our optimisation procedure for the assignment of pressure boundary condition has a potentially large number of solutions, we sought to determine the variability observed in vascular pressure predictions across multiple simulation runs. This experiment is described
in the Supplementary Information, and from which we found that the mean standard deviation of vascular pressure predictions, across simulation runs, was 0.25 mm Hg and 0.49 mm Hg in LS174T and SW1222 tumors, respectively. This is much smaller than the variability associated with spatial heterogeneity within the tumors (8.8 and 9.2 mm Hg, respectively).

**REANIMATE steady-state simulation in an orthotopic murine glioma model**

In order to evaluate the generalisability of the REANIMATE framework, we next applied it to data from orthotopic murine glioma tumors, derived from the murine GL261 cell line. At 20 days following the injection of glioma cells into the brain, tumors were resected from mice with a section of normal cortex tissue attached. Segmented tumor and cortex vessels are shown in Figure 4a, with brain vessels labelled in blue and tumor vessels in red.

As with colorectal tumors, steady-state vascular and interstitial REANIMATE fluid flow solutions were generated, which are shown in Figures 4b to 4f. The simulations predicted raised interstitial fluid pressure within the tumor (mean, 16 ± 10 mm Hg), and a mean interstitial perfusion of 1.3 ± 0.5 mL min⁻¹ g⁻¹. Comparison of these simulation results with in vivo measurements using ASL-MRI (shown in Figure 4d) revealed a good correspondence, with a mean measured tumor perfusion of 1.1 ± 0.7 mL min⁻¹ g⁻¹, and equivalent spatial distribution (hyperperfused periphery and central hypoperfusion). A scatter plot of the data is shown in Supplementary Figure 1c, which revealed a significant correlation between the two measurements ($r^2 = 0.91$, $p<0.001$, Pearson test).

Compared with subcutaneous tumors, REANIMATE predicted orthotopic gliomas to have a more uniform central IFP, which varied within a range of ±4 mm Hg. IFV was correspondingly low in the centre (<0.01 µm s⁻¹), and high at the periphery (indeed, much higher than in subcutaneous tumors (17 ± 4 µm s⁻¹)). No measurements of IFP or IFV exists in the literature to compare these data against, presumably due to the technical challenges associated with their measurement in deep-seated tumors.

In combination, these results demonstrate that mathematical modelling of fluid dynamics using optical image data from cleared tumor tissue as a substrate, is both feasible and provides quantitative predictions of vascular perfusion that are in keeping with experimental results. Therefore our next step was to use the steady-state flow predictions to parameterise a time-dependent model to simulate the delivery of exogenously-administered material.
As a first evaluation, we chose to model the dynamics of chelated gadolinium (Gd-DTPA), a widely used MRI contrast agent with well-studied pharmacokinetics, and directly compare them with \textit{in vivo} measurements. Gd-DTPA can be thought of as a proxy for a non-metabolised therapeutic agent. Time-dependent simulations were calculated using a ‘propagating front’ algorithm, that used steady-state solutions to mimic the physical delivery of material, both via vascular flow and by diffusion across blood vessel wall and through the interstitium.

To provide ground-truth data for comparison, and to generate new modelling substrates, we performed \textit{in vivo} experiments to measure the delivery of a bolus of Gd-DTPA in a set of LS174T (n=5) and SW1222 (n=6) tumors, using a dynamic contrast-enhanced (DCE) MRI sequence (Figure 5). Following these measurements, mice were culled via cervical dislocation. We resected and set two tumors aside (one LS174T and one SW1222) for processing within the REANIMATE framework.

Here, steady-state simulations were performed as described above, and were used as the basis for time-dependent delivery simulations. The influence of each vessel network inlet was modelled independently, and an algorithm was developed that monitored a propagating front through the network. Exchange between the vascular and interstitium was cast in a finite element framework, with vessel permeability (to Gd-DTPA flux) initially fixed at $1 \times 10^{-6}$ cm s$^{-1}$.\textsuperscript{22,23} The interstitium was modelled as a continuum with a constant cell volume fraction ($f_c = 0.8$).\textsuperscript{24} Gd-DTPA does not cross the cell membrane,\textsuperscript{25} and so Gd-DTPA concentration ([Gd]) was scaled by the fractional volume of the extra-cellular space, and we assumed a constant diffusion through the interstitium ($D = 2.08 \times 10^{-4}$ mm$^2$ s$^{-1}$.\textsuperscript{26}) Both \textit{in vivo} measurements and simulations had a duration of 12 minutes, with a temporal resolution of 16 seconds. Simulations were driven by a bi-exponential vascular input function, taken from the literature.\textsuperscript{27} Initially, 1% of the entire dose of the input function was partitioned across all tumor inlets, weighted by the inflow rate for the individual inlet.

REANIMATE intravascular and interstitial Gd-DTPA delivery predictions were rendered as videos (see \textbf{Supplementary Video 1} and \textbf{Supplementary Video 2}). Virtual sections from an LS174T tumor are also shown in Figure 5a, which revealed a prolonged, peripheral enhancement pattern, which is typical of the tumor type.\textsuperscript{20} In Figure 5b we show plots of contrast agent uptake, in which the greyscale coloring of each curve represents distance from the tumor edge (darkest at the edge, lightest in the centre). These reveal a highly
heterogeneous enhancement pattern, with decreasing concentration for increasing proximity to the tumor centre. Conversely, we found that SW1222 tumors enhanced with Gd-DTPA much more rapidly and homogeneously, with a peak enhancement at around 4 minutes, followed by a washout phase (see Supplementary Figure 2).

REANIMATE solutions describing Gd-DTPA delivery were analysed in the same manner as experimental data, i.e. as a function of distance from the tumor periphery. Simulations were performed in two stages: the first estimated Gd-DTPA enhancement using the initialisation parameter values defined above (the naïve solution); the second stage used modified parameter values, based on iteratively minimising the disparity between simulated and in vivo data.

Our naïve analysis underestimated the magnitude of contrast enhancement in LS174T tumors, but still reflected their spatial heterogeneity, with the $S_0$ parameter decreasing with distance from the tumor periphery. The enhancement rate parameter, $r_1$, provided a good fit to in vivo data, but did not reflect its increasing value at the tumor centre. To account for this, we increased the mean vascular permeability to $0.9\times10^{-6}$ cm s$^{-1}$ at the periphery, with a linear increase to $1.1\times10^{-6}$ cm s$^{-1}$ in the centre, which provided a better accordance with in vivo data (see Supplementary Figure 3). For the SW1222 tumor naïve simulation, contrast agent uptake was overestimated, but homogeneously distributed, reflecting what was found in vivo. The rate of enhancement was also much greater than in vivo. We therefore uniformly decreased vascular permeability to $0.75\times10^{-7}$ cm s$^{-1}$ in the second simulation, which then provided a good accordance with in vivo data ($p<0.01$, Kolmogorov-Smirnov). Scatter plots of the data, shown in Supplementary Figure 1d, also revealed a significant correlation ($r^2 = 0.92$, $p<0.001$, Pearson).

We can therefore conclude from these experiments that REANIMATE can provide good estimates of the delivery of Gd-DTPA, but which can be further improved by in vivo measurements.

Dual-fluorophore optical imaging of response to Oxi4503 treatment

Using our optimised Gd-DTPA delivery data, we went on to investigate the ability of REANIMATE to model drug uptake and response to treatment. This required the development of a dual-fluorophore imaging technique that allowed measurements of tumor vascular structure at two separate time points to be encoded. We chose to model vascular targeting therapy, due to its rapid, well-characterised mechanism of action, which can be
The acute effects of VDAs have been well-documented, using histology, MRI and in vivo confocal microscopy, which have demonstrated rapid vascular shutdown and extensive vessel fragmentation within the first 60 minutes to 24 hours of administration. This causes decreased perfusion, especially in the central part of the tumor, and an associated increase in hypoxia and cell death. In this study, we investigated a single dose of Oxi4503, at 40 mg kg\(^{-1}\).

Our dual-fluorophore method allowed us to characterise blood vessel structure at two separate time points, by administering fluorescently-labelled lectin (AlexaFluor-568) just prior to injecting Oxi4503, and then a second lectin 90 minutes later (AlexaFluor-647). Our rationale was that vessels occluded by Oxi4503, and were no longer perfused, would be labelled by only the first fluorophore; vessels that remained perfused following therapy would be labelled with both fluorophores. As a validation of our results, in vivo arterial spin labelling (ASL) MRI was also performed on a subset of tumors (n=3 of each tumor type).

Mice, each bearing an LS174T or SW1222 tumor, were randomly assigned to treatment (Oxi4503, 40 mg kg\(^{-1}\)) or control groups (administered saline). Figure 6a shows example volume renderings of dual-stained vessel networks, in which vessels were colored blue if co-labelled with both fluorescent lectins (i.e. vessels that were perfused both pre- and at 90 minutes post-Oxi4503 administration) or green if perfusion was evident pre-treatment but had been removed at 90 minutes. See Supplementary Video 3 for a three-dimensional rendering of the data.

Figures 6b to 6d document the effect of Oxi4503 on the geometry of LS174T and SW1222 vessel networks. Figure 6b shows a graph comparing the mean distance of blood vessels from the centre of each tumor type, pre- and post-treatment with Oxi4503. This plot shows that, for LS174T tumors, vessel that became non-perfused with Oxi4503 (i.e. vessels rendered in green) were generally located in the centre of tumors, whereas SW1222 displayed a more distributed and localised pattern of perfusion loss. In Figure 6c we show a plot of the number of graphical nodes within each cluster that became isolated by this loss of perfusion, which was significantly different between LS174T and SW1222 tumors (LS174Ts displayed much larger clusters (P<0.01)). This further demonstrates the fragmented nature of the SW1222 tumors’ response to Oxi4503.

To ensure that these changes in vascular geometry were induced by the action of Oxi4503, blood volume was measured pre- and post-Oxi4503 and compared against control groups that received saline only. As can be seen in Figure 6d, Oxi4503 induced a significant decrease
in both tumor types (P<0.01), but in control tumors, no significant change was found (p>0.05).

**REANIMATE simulations of response to Oxi4503**

Having identified significant differences between SW1222 and LS174T tumors in their vascular structural response to Oxi4503, we next aimed to use REANIMATE to simulate changes in tumor perfusion and IFP induced by Oxi4503, to attempt to further explore these differences. **Figures 7a-c** show the results of REANIMATE simulations of vascular flow, IFP and interstitial perfusion in an example LS174T tumor, pre- and post-Oxi4503. Each reveals a spatially heterogeneous response to the drug, with both increases and decreases in perfusion and IFP observed within the same tumors, representing a redistribution of flow in response to localised vascular occlusion.

These trends were replicated in *in vivo* ASL data (example images are shown in **Figure 7d**) which measured a significant decrease in median tumor perfusion of 9.8% (from 0.61 to 0.55 mL min⁻¹ g⁻¹, p<0.05) in LS174T tumors, but which was accompanied by a significant *increase* in the 90th percentile perfusion value (from 2.48 to 2.64 mL min⁻¹ g⁻¹, P<0.01). Our REANIMATE simulations also predicted a decrease in IFP of 4.5 mm Hg, but accompanied by an increase of 3.6 mm Hg in the 90th percentile. These results demonstrate a complex redistribution of flow caused by the vascular disrupting agent at this early time point, which we sought to better understand. In SW1222 tumors, *in vivo* measurements of perfusion and IFP did not significantly change. Moreover, the fragmented nature of dual-labelled fluorescence images meant that post-Oxi4503 steady-state simulations could not be performed in SW1222 tumors.

Our first REANIMATE computational experiment aimed to simulate the uptake of Oxi4503, using a similar approach as taken for Gd-DTPA uptake simulations, but over a longer duration (90 minutes, with a temporal resolution of 10 seconds). Oxi4503 has a molar mass of 332.35 g mol⁻¹ (approximately one third that of Gd-DTPA), so, using the Stoke-Einstein relation, \( D = \frac{7.37 \times 10^{-5}}{\text{mm}^2 \text{ s}^{-1}} \). Systemic pharmacokinetics for Oxi4503 were taken from the literature, \(^{34}\) and expressed as an exponential decay function (Equ. 15). Both intravascular and interstitial drug concentrations were simulated, and results are shown in **Figure 8a** (see, **Supplementary Video 4** for a four-dimensional representation). As with Gd-DTPA experiments, Oxi4503 uptake was spatially heterogeneous.
We then used these REANIMATE simulations of Oxi4503 uptake to test two hypotheses: 1) that vessels that receive the greatest Oxi4503 exposure are more likely to become non-perfused; 2) that network geometry differences between tumor types could influence their response to VDA therapy.

To test the first hypothesis, we compared vessels from our dual-labelled datasets that had lost perfusion post-Oxi4503 (i.e. were labelled with just one fluorophore), with their simulated exposure to Oxi4503, as predicted by REANIMATE. The box graph in Figure 8b displays the result of this analysis, in which nodes connecting only non-perfused ('green') vessels had a significantly lower exposure to Oxi4503 than nodes connecting a mixture of non-perfused and perfused ('blue') vessels (P<0.05). Similarly, Figure 8c and 8d show the location of perfused and non-perfused vessels and the cumulative exposure to Oxi4503 at 90 minutes post-administration, which shows non-perfused regions with low Oxi4503 exposure; on average non-perfused vessels exhibited a significantly lower simulated exposure than vessels that remained perfused (2.0 compared with 3.8 mM min m⁻² (p<0.001)). These results are inconsistent with our first hypothesis, that perfusion loss would be associated with greater Oxi4503 exposure, and so the hypothesis was rejected. This lead us to next evaluate our second hypothesis, and investigate differences in the vascular architecture of the two tumor types. In particular, we evaluated the functional connectivity of the two tumor types.

Functional (or logical) connectivity and redundancy measures describe the connectedness of individual vessel networks, following pathways of decreasing fluid pressure. Specifically, redundancy was measured by $N$, the mean number of viable alternative pathways for each node if the shortest path (based on flow velocity) were occluded, and $r$, the average additional distance that would be travelled. Connectivity was defined as the sum of the number of nodes upstream and the number of nodes downstream of a given node, divided by the total number of nodes in the network.

Figures 9a and b show histograms comparing log($C$) and $r$ measurements in LS174T and SW1222 tumors. These data revealed that SW1222 tumors had significantly greater vascular connectivity than LS174T tumors ($C = 0.15 ± 0.06$ and $0.06 ± 0.05$, respectively) (P<0.01). They also display greater redundancy, with $N = 1.9 ± 0.9$ and $1.5 ± 0.7$ and $r = 1.02 ± 0.02$ and 1.04 +/- 0.05, for SW1222 and LS174T tumors, respectively. Regional connectivity was also mapped, with examples shown in Figures 9c (LS174T tumor) and d (SW1222 tumor), in which nodes are scaled and color-coded according to their connectivity measure. These
clearly show the greater connectivity evident in this example SW1222 tumor, than in the
LS174T tumor.

Referring back to the definitions of connectivity and redundancy above, these results
suggest that, in LS174T tumors, vessels that become non-perfused due to targeting by a high
concentration of Oxi4503 can cause large vascular territories downstream to become non-
perfused, due to their lack of connectivity. In SW1222 tumors, loss of perfusion can be
compensated for by rerouting flow through alternative routes, thanks to their high
redundancy. This explanation, which requires further evaluation, would explain the
different pattern of response observed in SW1222 and LS174T tumors, with LS174T tumors
showing large regions of perfusion loss in dual-fluorophore data (particularly in their core),
whilst SW1222 show a more distributed pattern with flow loss in individual vessels.

Discussion

Computational modelling of cancer has a relatively long history,\textsuperscript{35-39} and has provided
valuable improvements in our understanding of the development and treatment of cancer.
As noted by Altrock, Liu and Michor, “the power of mathematical modelling lies in its ability
to reveal previously unknown or counterintuitive physical principles that might have been
overlooked or missed by a qualitative approach to biology”.\textsuperscript{39} Successes have been found in
a range of areas, from modelling the dynamics of mutation acquisition\textsuperscript{40} to multiscale
modelling of the interaction between tumor cells and their microenvironment.\textsuperscript{41} Previous,
seminal studies have combined experimental and numerical approaches to study the
relationship between tumor microstructure and the delivery of therapeutic agents.\textsuperscript{36,37,42} A
key observation from this work was that the chaotic organisation of tumor blood vessels,
their highly permeable vessel walls and missing or non-functioning lymphatics can result in
elevated interstitial fluid pressure (IFP) and limited blood flow. Both of these effects,
alongside high cell density and thick extracellular matrix, can conspire to limit the delivery of
systemically administered therapeutic agents, and can therefore act as a source of
therapeutic resistance.\textsuperscript{43}

Whilst these principles are well established, the complex manner in which these phenomena
can interact and vary within real-world tumors, and how individual drugs of different sizes
and physico-chemical properties are distributed, is not well understood. This points to a
critical need for better understanding of drug delivery to solid tumors, which could, at least
in part, help to address the pharmaceutical industry’s current low approval rate for new
cancer therapies, for which the influence of delivery is often an overlooked factor.\textsuperscript{44,45} Equally, this knowledge could provide improvements in the clinical management of the wide range of tumor types encountered in the clinic and enable treatments to be more effectively personalised. This is an example of an area in which we believe our REANIMATE framework will find wide application.

In this study, we introduced and provided a first demonstration of the application of REANIMATE, which is a large-scale, three-dimensional imaging, modelling and analysis framework, which uses data from optical imaging of cleared tissue\textsuperscript{16,46,47} to produce realistic substrates for computational modelling of fluid dynamics in the tumor microenvironment, optionally guided by in vivo imaging data. We applied REANIMATE to imaging data from murine models of colorectal cancer and glioma to simulate: 1) steady-state fluid dynamics (blood flow, intravascular and interstitial fluid pressure); 2) uptake of the MRI contrast agent Gd-DTPA; and 3) uptake and response to vascular-targeting treatment (Oxi4503). Our results demonstrated the feasibility and accuracy of this whole-tissue approach to numerical modelling, which allows computational experiments to be performed on real-world tumors. A key advantage of this approach is the ability to directly compare modelling solutions with experimental measurements from the same tumors.

Whilst, in principle, any number of physiological phenomena could be incorporated into the REANIMATE framework, we have initially focussed on modelling intravascular and interstitial delivery. This used well-established biophysical models, but on a larger scale than has previously been undertaken, and using real-world vascular networks. A justification for simulations at this scale is provided by the multi-scale interactions evident in tumor fluid dynamics. For example, elevated IFP is maintained via a whole-tumor distribution of both vascular perfusion and interstitial drainage; likewise, perfusion is spatially heterogeneous, meaning that one tumor sub-region can be very different to another.

Previous studies have used mathematical modelling approaches to study tumor blood and interstitial flow, and have often focussed on their associated spatial heterogeneity.\textsuperscript{48} For example, Baxter and Jain\textsuperscript{36,42} described raised, homogeneous interstitial fluid pressure at the centre of tumors, which drops precipitously at the tumor periphery. This result was based, in part, on simulations of spherical, spatially-homogeneous tumor vasculature. Numerous studies have subsequently incorporated more realistic vasculature into models stemming from Baxter and Jain’s work, such as synthetically-generated vascular networks,\textsuperscript{49-53} using micro-CT data from microvascular casts to model intravascular blood flow,\textsuperscript{54} or using
subnetworks from tumors, derived from imaging data.\textsuperscript{55-57} Synthetically-generated vasculature, using angiogenesis models, have been used to formulate hypotheses on the delivery of chemotherapeutics\textsuperscript{58,59}, investigate the impact of tumor size on chemotherapeutic efficacy\textsuperscript{60} and to investigate the effect of dynamic vasculature\textsuperscript{61} and the structure and morphology of vascular networks\textsuperscript{62} on drug delivery.

REANIMATE builds on and extends this work by including simulation substrates from complete, real-world tumors, in three spatial dimensions, which are guided by and compared against \textit{in vivo} measurements. In this initial demonstration, we focussed on the vasculature of colorectal xenograft models, which we imaged by labelling with fluorescent lectin, allowing blood and interstitial flow to be explicitly simulated in realistic networks. We treated the interstitium as a continuum, but future generations of the framework could include additional structural elements such as cell membranes and nuclei, by multifluorescence labelling. Indeed, there is significant potential for extending and enhancing REANIMATE in other pathologies, to allow more in-depth computational experiments to be performed.

A key advantage of REANIMATE is its ability to compare model predictions with experimental measurements from the same tumors. We found a good correspondence between our predictions of vascular perfusion and delivery of Gd-DTPA, and those from \textit{in vivo} imaging, both in their magnitude and spatial distribution. Perfusion was predicted to be significantly greater in SW1222 tumors than in LS174T tumors, which reflects the results of \textit{in vivo} arterial spin labelling measurements, and was highly heterogeneous, with flow concentrated at the periphery of both tumor types. Conversely, Gd-DTPA uptake was more heterogeneous in LS174T than SW1222 tumors, both in \textit{in vivo} MRI measurements and simulations (with minimal parameter optimisation).

Through its use of real-world, whole tumor substrates, REANIMATE undertakes modelling at a whole-tissue scale. In many organs, this would enable pressure boundary conditions to be defined in a straightforward manner, potentially by directly measuring inlet and outlet pressures. However, subcutaneous tumor xenograft models normally exhibit a large number of small feeding vessels, and so explicitly measuring and defining pressure boundary conditions in this context is challenging. Our pressure boundary condition optimisation procedure was a pragmatic solution to this problem, and enabled the use of target average pressures. We found that the variability of pressure predictions (from which all other parameters are ultimately derived), was much lower than the variability associated with
spatial heterogeneity within the tumor. However, this approach could still provide a source of error and, whilst our model solutions agreed well with experimental measures, better approaches could doubtless be developed. For example, as suggested above, the use of tumors with a small number of well-defined inlet and outlet vessels, with measurable pressures, would be advantageous, and the use of complete tumor vascular networks would enable such an approach to be realised.

In summary, the results of this study show that, by adding realistic, whole-tumor microstructure, with its inherent heterogeneity, accurate predictions for tumor fluid dynamics and material delivery to be made. These results are important as Gd-DTPA can be thought of as a proxy for delivery of a (non-metabolised) drug, enabling the accuracy of REANIMATE delivery predictions to be verified. Indeed, REANIMATE could easily be modified to include terms for metabolism, and acute response by the microenvironment could be modelled by modifying cell density terms. This could therefore allow panels of drugs to be assessed, with different delivery characteristics, to predict candidates that are most (or least) likely to achieve a therapeutic response.

To further investigate this ability, we used REANIMATE to study the response of our colorectal xenografts to Oxi4503 treatment, and showed that structural connectivity and redundancy in colorectal tumor xenograft model vascular networks can introduce different responses to the vascular-targeting agent Oxi4503. SW1222 tumors, with their greater connectivity and redundancy, are more able to resist loss of flow in individual vessels, by rerouting flow via local pathways, whereas there is much greater potential for LS174T tumors to lose perfusion in large downstream subnetworks. These results reflect those that we have previously observed in vivo when assessing the response of colorectal metastases to Oxi4503 treatment in the liver, in which the magnitude of the response decreased with increasing distance of individual tumors from major blood vessels. From a computational modeling perspective, these results are also important, as they demonstrate a mechanism through which tumors can become resistant to drug therapies, and which manifests via complex interactions across large regions within a tumor, or across whole organs.

These results each demonstrate the important potential role for large, realistic tumor simulations, of the form developed here with REANIMATE. The detailed insights generated in this study could not have been made with conventional two-dimensional analysis of histological sections, or in vivo experiments that lack the spatial resolution and functional information to access this information, and demonstrates a key strength of the REANIMATE
We anticipate that REANIMATE will enable us to further study and understand complex interactions between biological phenomena, allowing new insights into key challenges in cancer research. Whilst the limitations to drug delivery caused by the physiological structuring of tumors have been well-studied, REANIMATE could enable a better understanding of limitations in tumor drug delivery in individual tumors (and how this can be mediated) and the development of resistance to therapy via physical (rather than biochemical) mechanisms. Moreover, if applied to biopsy samples, or resected, intact tumors (or tumor deposits), could provide useful insights into treatment stratification in the clinic.

**Acknowledgements**

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**Author Contributions**

Ad’E designed and performed optical imaging experiments, analysed and interpreted results and wrote the first drafts of the manuscript. PS and RS developed software to perform mathematical and computational analysis. PS analysed and interpreted data and wrote and edited the paper. MA performed a subset of the optical imaging experiments. MS and SWS developed software for performing time-dependent simulations. RR and TAR designed and performed ASL-MRI measurements, and developed software for quantifying the data. GA provided murine brain tumor models. AD assisted in student supervision and design of optical imaging experiments. MFL provided access to imaging resources and student supervision. RBP provided murine xenograft models and interpreted results. SWS developed software for segmenting optical imaging data. RS and SWS secured funding, supervised the design of experiments and simulations, developed the main concepts, interpreted results, and contributed to the writing and editing of the manuscript.

**Competing Interests**

The authors declare no competing interests.
**Data availability**

Raw data generated from this study can be found at http://doi.org/10.17605/OSF.IO/ZH9EU

**Code availability**

Code used to analyse blood vessel networks in Python 2.7 is available in a GitHub repository, and available under BSD licence: https://github.com/CABI-SWS/reanimate. Code used to model tumor fluid dynamics in C++ is also available in a separate GitHub repository: https://psweens.github.io/VF_NatureBioEng/

**Materials and Methods**

**Tumor xenograft models**

All experiments were performed in accordance with the UK Home Office Animals Scientific Procedures Act 1986 and UK National Cancer Research Institute (NCRI) guidelines. Female, 8 week old, female, immune-compromised nu/nu nude mice (background CD1) were used throughout this study (Charles River Laboratories). Human colorectal adenocarcinoma cell lines (SW1222 and LS147T) were cultured in complete media (Minimum Essential Medium Eagle with L-Glutamine (EMEM) (Lonza, Belgium) + 10% fetal bovine serum (Invitrogen, UK)) in a ratio 1:20 (v/v) and incubated at 37 °C and 5% CO₂. To prepare for injection, cells were washed with DPBS and detached with trypsin-EDTA (7-8 min, 37 °C, 5% CO₂). A 100 µl bolus of 5x10⁶ cells was injected subcutaneously into the right flank above the hind leg. Tumor growth was measured daily with callipers, for between 10 to 14 days.

**Orthotopic glioma models**

Female, 8 week old, C57BL/6 mice were injected with 2x10⁴ GL261 mouse glioma cells. Mice were anesthetized with 4% isoflurane in an induction box and then transferred to a stereotactic frame (David Kopf Instrument, Tujunga, CA), where anaesthesia was delivered through a nose cone and maintained at 2%. The head was sterilised with 4% chlorhexidine and the skin was cut with a sterile scalpel to expose the skull. Coordinates were taken using a blunt syringe (Hamilton, 75N, 26s/2½/3, 5 µL): 2mm right and 1mm anterior to the bregma, corresponding to the right caudate nucleus. A burr hole was made using a 25-gauge needle. The Hamilton syringe was lowered 4mm below the dura surface and then retracted by 1mm.
to form a small reservoir. 2x10^4 GL261 cells were injected in a volume of 2 μL over two minutes. After leaving the needle in place for 2 minutes, it was retracted at 1 mm/min. The burr hole was closed with bone wax (Aesculap, Braun) and the scalp wound was closed using Vicryl Ethicon 6/0 suture.

Fluorescent labelling of tumor vasculature and perfusion fixation

Lectin (griffonia simplicifolia) bound to either Alexa-647 (Thermo Fisher Scientific, L32451) or Alexa-568 (Thermo Fisher Scientific, L32458) was injected intravenously (i.v.) and allowed to circulate for 5 minutes, prior to perfuse fixation, to allow sufficient binding to the vascular endothelium.²

To prevent blood clot formation within the vasculature, mice were individually heparinized by intraperitoneal (i.p.) injection (0.2 ml, with 1000 IU ml⁻¹). Mice were terminally anaesthetized by i.p. injection of 100 mg kg⁻¹ sodium pentobarbital (Animalcare, Pentoject) diluted in 0.1 ml phosphate buffered saline (PBS). Once anaesthesia was confirmed, surgical procedures for intracardial perfusion were performed for systemic clearance of blood. PBS (30 ml, maintained at 37 °C) was administered with a perfusion pump (Watson Marlow, 5058) at a flow rate of 3 ml/min to mimic normal blood flow. After the complete drainage of blood, 40 ml of 4% paraformaldehyde (PFA, VWR chemicals) was administered. Harvested tumors were stored for 12 hours in 4% PFA (10 ml total volume, at 4 °C).

Treatment with Oxi4503

Following 10 to 14 days of growth, mice were randomly assigned to treatment (Oxi4503, n=6) and control (saline) groups, with n=3 SW122 and n=3 LS174T in each. Treated groups were injected i.v. with 100 μg lectin-AlexaFluor 647 diluted in sterile saline at neutral pH (100 μl) containing 1 mM CaCl₂, followed by administration of OXi4503 (40 mg kg⁻¹, 4 mg ml⁻¹). Control mice were injected with 100 μl saline. After 2 hours, all mice were injected i.v. with 100 μg lectin-AlexaFluor 568 diluted in sterile saline at neutral pH (100 μl) containing 1 mM CaCl₂. 5 minutes after injection mice were culled and underwent perfuse-fixation, as described above.

Optical clearing and imaging

Following perfuse-fixation, tumors were resected and rinsed three times in PBS, for 10 minutes each, prior to clearing, to remove residual formaldehyde and avoid over-fixation. After PBS rinsing, harvested tumors were optically cleared with BABB (1:2 benzyl alcohol:...
benzyl benzoate). Our BABB clearing preparation consisted of dehydration in methanol for 48 hours followed by emersion in BABB for 48 hours.

Fluorescently-labelled tumor vasculature in cleared tissue was visualized with optical projection tomography (OPT, Bioptomics, MRC Technologies, Edinburgh). Lectin-AlexaFluor 647 was imaged using a filter set with excitation range 620/60 nm, and emission 700/75 nm. For vessels labelled with lectin-AlexaFluor 568, a filter set with excitation 560/40 nm and emission LP610 nm was used. Measurements were performed with an exposure time of 1600-2000 ms for lectin-AlexaFluor 647 and of 270-600 ms for lectin-AlexaFluor 568, which was varied according to sample size. The rotation step was 0.45 degrees. The final resolution ranged from 4.3 µm to 8 µm, depending on the sample size.

OPT data were reconstructed with Nrecon (Bruker, Ettlingen, Germany). Misalignment compensation was used to correct misalignment during projection image acquisition, in order to reduce tails, doubling or blurring in the reconstructed image. Depth of correction for ring artefact reduction was 4 and defect pixel masking was 50% for all scans.

Image processing and vessel segmentation

Reconstructed OPT data were used to generate whole-tumor blood vessel networks. Firstly, a three-dimensional Gaussian filter with a width of 50 pixels (corresponding to a physical size of 300 µm, greater than the largest vessel diameter) was applied. The filtered data were subtracted from the original data to remove background variations in autofluorescence. A three-dimensional Frangi filter was then applied (Matlab, MathWorks, Natick, MA) to enhance vessel-like structures. The response to the filter was thresholded to segment blood vessels from background. Skeletonisation of these thresholded data was performed in Amira (Thermo Fisher Scientific, Hillsboro, OR), which also converted the data into graph format (i.e. nodes and segments with associated radii). To ensure that vessel structures were accurately represented, 2D sections from the original image data were swept through reconstructed 3D networks (in Amira), with visual inspection used to for an accordance between vessel location and thickness, and the location of fluorescence signal.

Mathematical model of steady-state tissue fluid dynamics

Blood flow through the segmented vascular network was modelled by Poiseuille’s law, using empirically-derived laws for blood viscosity (assuming constant network haematocrit) and following the established approach developed in and applied to numerous tissues (for
example mesentery, muscle, cortex, and tumours. This model assumes conservation of flux at vessel junctions to define a linear system to solve for the pressures at nodal points in the network (from which vessel fluxes are calculated using Poiseuille).

Boundary conditions on terminal nodes in the network were estimated using the optimisation method of Fry et al., which matches the network solution to target mean shear stress and pressure values.

The approach of Fry et al. requires a proportion of boundary conditions to be applied to a microvascular network. However, neither flow or pressure measurements were obtained in individual vessels in vivo for our tumor networks. As such, an optimisation procedure was employed to induce a physiological pressure drop (55 to 15 mmHg for both LS147T and SW1222 simulations) across peritumoral boundary vessels in a network. Consistent with previous studies, 33% of internal nodes were assigned zero flow with all remaining boundary nodes determined using the optimisation algorithm of Fry et al. This procedure was repeated until simulations ensured physiologically realistic tissue perfusion when compared to that gathered in vivo using ASL MRI.

The network flow solution was coupled to an interstitial fluid transport model, adapting the approach taken in Secomb et al. to model oxygen delivery to tissue. The interstitium is modelled as a porous medium using Darcy’s law,

\[ \mathbf{u} = -\kappa \nabla p, \]  

subject to \( p \to p_I \) as \( |x| \to \infty \). Here, \( \mathbf{u} \) is the volume-averaged interstitial blood velocity (IFV), \( p \) is the interstitial fluid pressure (IFP), \( p_I \) is the target IFP, and \( \kappa \) is the hydraulic conductivity of the interstitial tissue. Starling’s law is used to describe fluid transport across the endothelium, from the vessels into the interstitium:

\[ q = L_p S \cdot (\Delta p - \sigma \Delta \Pi), \]  

where, \( q \) is the fluid flux across the endothelium, \( L_p \) is the hydraulic conductance of the vessel wall, \( S \) is the surface area of the vasculature, \( \sigma \) is the oncotic reflection coefficient and, \( \Delta p \) and \( \Delta \Pi \) fluid and oncotic pressure gradients between the vasculature and tissue.

To solve the model computationally, we discretized the tumor vasculature into a series of \( M \) sources of strength \( q_{s,j} \) so that the conservation of mass equation is modified to

\[ -\kappa \nabla^2 p = \sum_{j=1}^{M} q_{s,j}(x) \delta(x - x_j), \]  

where \( x_j \) and \( q_{s,j} \) are the spatial coordinates and (unknown) strength at \( x_j \) of source \( j \),
respectively, and \( \delta(x-x_j) \) is the three-dimensional delta function. An axisymmetric Greens solution, \( G(r) \) where \( r=|x-x_j| \), was sought for equation 3 subject to the boundary condition that \( p \to p_t \) as \( |x| \to \infty \), motivated by distributing the delta function \( \delta(x-x_j) \) uniformly over a sphere of finite radius \( r_0 \) (set to the radius of blood vessel \( j \)), the solution to equation 3 may be approximated by

\[
G_{ij} = \begin{cases} 
\frac{3-\left(\frac{r_{ij}}{r_0j}\right)^2}{8\pi\kappa r_{ij}}, & \eta_{ij} \geq r_0j \\
\frac{1}{4\pi\kappa r_{ij}}, & \eta_{ij} < r_0j 
\end{cases}
\]

where \( r_{ij} = |x_i - x_j| \) is the distance between sources \( i,j \in M \). The corresponding interstitial fluid pressure (IFP) at source \( i \) may be approximated by

\[
p_i = p_t + \sum_{j=1}^{N} G_{ij} q_{S,j}, \quad \text{for } i \in M. \tag{5}
\]

Assuming flux of fluid across the wall of vessel \( i \), \( q_{V,i} \), is continuous yields

\[
q_{V,i} = -2\pi \kappa r_{v,i} l_i \sum_{j=1}^{M} \nabla G_{ij} q_{S,j}. \tag{6}
\]

Starling’s law, equation 2, can be written in the form

\[
p_{V,i} = p_{b,i} - K q_{V,i} - \sigma (\Pi_{b,i} - \Pi_{V,i}), \quad \text{for } i \in M, \tag{7}
\]

where \( p_{V,i} \) and \( \Pi_{V,i} \) are the blood and oncotic pressure at the vessel wall, \( p_{b,i} \) and \( \Pi_{b,i} \) is the intravascular blood pressure in the absence of diffusive interstitial fluid transfer (calculated using the Poiseuille flow model) and oncotic pressure, and \( q_{V,i} \) is the rate of fluid flow per unit volume from blood vessel \( i \) to the interstitium. The intravascular resistance to fluid transport, is defined by \( K = 1/L_p S \).

Equations 5, 6 and 7 were combined to form a dense linear system, which was solved to give the IFP field throughout the tissue. Parameter values for the complete mathematical model are shown in Supplementary Table 2.

**Mathematical model of time-dependent vascular and interstitial transport**

A ‘propagating front’ (PF) algorithm was developed to describe the transport of solute (e.g. a drug) through the tumor vessel network and interstitium. This model considers the timescale for delivery of a drug, on which the flow problem is assumed to be steady (the timescales for drug transport by advection and diffusion are much faster than those for vascular adaption, which would contribute to a non-steady flow solution). A vascular input function was first defined, which describes the time-dependent delivery of the drug concentration into the network, and which then propagates throughout the network.
according to the network topology and flow solution. The influence of each vessel network inlet was modelled independently and each solution linearly superimposed, allowing the algorithm to be parallelised.

Each node was assigned a set of values, \( J \) describing the ratio of the flow in each vessel segment connected to the node \( (F) \) to the total inflow into the node \( (F_a) \). Flow values were taken from the steady-state model defined above. The \( J \) values were propagated through the network, following pathways with decreasing vascular pressure. Using velocities from the steady-state solution, delays \( (d) \) were also assigned to each vessel segment. Vessel segments attached to each node were categorised as outflows (negative pressure gradient) or inflows (positive pressure gradient). Time-dependent drug concentration in the \( k^{th} \) outflowing vessel segment \( (C_k(t)) \) was modelled as

\[
C_k(t) = J_k \sum_{j=1}^{N} C_j(t - d_k), \tag{8}
\]

where \( C_j(t) \) is the concentration in the \( j^{th} \) inflowing vessel segment and \( N \) is the total number of inflowing vessel segments.

Interstitial delivery was cast in a forward finite difference framework, in which vessels were considered as radial emitters. Points were gridded on concentric cylinders, regularly spaced around the vessel segment (with spacing ranging from 10 to 100 µm). Exchange of the drug across the vessel wall and diffusion through the interstitium were modelled as

\[
C^I_{j+1}(r) = AC^I_j(r) + \Gamma (C^V_j - C^I_j(r)), \tag{9}
\]

\[
C^V_{j+1} = \sum_{j=1}^{N} \Gamma (C^I_j - C^V_j), \tag{10}
\]

where \( C^I_{j+1} \) is the interstitial concentration at the \( j^{th} \) time point (at a radial distance \( r \) from the vessel) and \( C^V_j \) is the vascular concentration.Interstitial velocity and pressure were not used in the time-dependent model, for simplicity, but could be incorporated in future studies, which could be particularly relevant for simulating the delivery of large molecules. The coefficient \( A \) is a two-dimensional square matrix of dimension \( n \), where \( n \) is the number of radial positions in the interstitial finite difference calculation, \( h \) is their radial separation and \( k \) is the spacing between time steps:

\[
A = \begin{bmatrix}
1 - 2\lambda & \lambda & 0 & 0 & 0 \\
\lambda & 1 - 2\lambda & \lambda & 0 & 0 \\
0 & \lambda & 1 - 2\lambda & \lambda & 0 \\
& & & \ddots & \ddots \\
0 & 0 & 0 & 0 & \lambda & 1 - 2\lambda \\
\end{bmatrix}, \tag{11}
\]

\[
\lambda = \frac{Dk}{h^2} \tag{12}
\]
Here $D$ is the diffusion coefficient of the agent under investigation. Following each finite difference step, interstitial diffusion solutions were regridded to a course 64×64×64 matrix (approximately 100 µm isotropic resolution) for storage. During regridding, molar quantities were converted to molar concentration, with the parameter $\Gamma$ controlling the transport across the vessel wall,

$$\Gamma = \frac{L_p S}{VQn}$$  \[13\]

in which, for small molecules, transport was assumed to be diffusive, and pressure terms were assumed to be negligible.

**Measurement of vessel network functional connectivity and redundancy**

The mean number of viable alternative pathways, $N$, for each node if the shortest path (based on transit time – i.e. incorporating flow velocity) was occluded was used to define the redundancy of tumor vessel networks, alongside $r$, the average additional distance that would be travelled.\textsuperscript{81} Connectivity was defined as the sum of the number of nodes upstream and the number of nodes downstream of a given node, divided by the total number of nodes in the network. All three measures reflect functional connectivity (i.e. following pathways with decreasing vascular pressure from steady-state fluid dynamics simulations), and were estimated from vessel networks using algorithms written in-house in Python 2.7.

**Simulation of Gd-DTPA delivery**

The systemic pharmacokinetics for Gd-DTPA in mice, following an i.v. bolus injection, were modelled as a biexponential decay:

$$C^v(t) = a_1 e^{-m_1 t} + a_2 e^{-m_2 t}$$  \[14\]

with $a_1 = 2.55$ mM, $m_1 = 8 \times 10^{-2}$ s$^{-1}$, $a_2 = 1.2$ mM and $m_2 = 1 \times 10^{-3}$ s$^{-1}$.\textsuperscript{27}

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**Simulation of Oxi4503 delivery**

Oxi4503 systemic pharmacokinetics were modelled as a single exponential function, of the form

\[
C^V(t) = C_{\text{max}} e^{-R_{1/2} t}
\]  

[15] with \( R_{1/2} = 3.1 \times 10^{-5} \text{ s}^{-1} \) and \( C_{\text{max}} = 7.7 \mu \text{M} \). This assumed a mouse mass of 25 g and injection dose of 40 mg kg\(^{-1}\).

**Dynamic contrast-enhanced MRI**

Gadolinium-DTPA (Magnevist, Bayer, Leverkusen, Germany) was injected as a bolus into mouse tail veins, using a power injector (Harvard Instruments, Cambourne, UK). We injected 5 mL kg\(^{-1}\) over a period of 5 seconds, which was initiated at 90 seconds after the start of a dynamic, spoiled gradient-echo sequence (TE, 2.43 ms; TR, 15 ms; flip angle 20°; 5 slices; slice thickness 0.5 mm; matrix size, 128×128; FOV, 35×35 mm; temporal resolution 16 s; total duration 15 minutes). The change in signal intensity induced by contrast agent was calculated by subtracting the mean signal from the first 5 frames from the acquisition.

Signal intensity was converted to gadolinium concentration, via the change in longitudinal relaxation rate \( R_1 \) and contrast agent relaxivity (\( c_1 \) fixed at 2.9 mM\(^{-1}\) s\(^{-1}\)):

\[
C(t) = \frac{R_1(t) - R_{10}}{c_1}
\]  

[16] \( R_1(t) \) was estimated from the theoretical change in spoiled gradient-echo signal magnitude.  

\( R_{10} \) was the mean, pre-enhancement \( R_1 \), which was estimated from a Look-Locker multi-
inversion time acquisition, acquired prior to the dynamic sequence (TE, 1.18 ms; inversion time spacing, 110 ms; first inversion time, 2.3 ms; 50 inversion recovery readouts).

Contrast agent uptake data were fitted to a phenomenological model of the form

\[ C(t) = S_0 (1 - e^{-r_2(t-t_0)}) e^{-r_2(t-t_0)} \]  \[17\]

where \( S_0, r_1, r_2 \) and \( t_0 \) were fitted parameters. Fitting was performed in Python 2.7 (leastsq algorithm from the scipy package).

Arterial spin labelling MRI

We acquired arterial spin labeling (ASL) data with a flow-sensitive alternating inversion recovery (FAIR) Look-Locker ASL sequence, with a single-slice spoiled gradient echo readout (echo time, 1.18 ms; inversion time spacing, 110 ms; first inversion time, 2.3 ms; 50 inversion recovery readouts; 4 averages). Regional perfusion maps were calculated as described by Belle et al. (38), with an assumed blood-partition constant of 0.9.

Statistics

Differences between groups were tested for significance with the non-parametric, two-sided Wilcoxon rank sum test (Python 2.7, scikit package). \( P < 0.05 \) was considered significant. All summary data are presented as mean ± SD.

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**Figure Captions**

**Figure 1.** The REANIMATE pipeline for *in vivo* and *ex vivo* imaging of intact tumors and performing three-dimensional computational fluid mechanics simulations. After *in vivo* imaging (1), which can be performed longitudinally during tumor growth, tumors are resected and optically cleared (2), to render tumors transparent for three-dimensional fluorescence imaging. Optical images are processed to segment fluorescently-labelled structures within the tumor microenvironment (3) (in this case, blood vessel networks), which are reconstructed in 3D graph format (nodes and connecting segments, each with a radius corresponding to the size of the blood vessel). These geometrical data become the substrate for computational fluid dynamic models to estimate steady-state blood flow and interstitial transport (4) and time-dependent numerical modelling of drug delivery (5). All of these data can then be used to perform *in silico* experiments (e.g. assessing the heterogeneous delivery of drugs or contrast agents), which can be compared with *in vivo* experiments in the same tumor models, or even the same mice. In this study, REANIMATE is used to study the action of a vascular disrupting agent (Oxi4503) in two models of human colorectal carcinoma.

**Figure 2.** Three-dimensional blood vessel networks, segmented from optical imaging data acquired from complete colorectal carcinoma xenografts, and reconstructed in graphical format (diameters scaled according to their measured values). a) and b) show reconstructed networks from LS174T and SW1222 tumors, respectively, with inset panels showing zoomed-in regions. The two tumor types displayed significant differences in vascular architecture, as well as intra-tumor spatial heterogeneity. c) and d) show example LS174T vessel networks overlaid on raw image data. A good accordance can be seen between hyper-intense vessel structures in the image data and graphical format vessels.

**Figure 3.** REANIMATE, steady-state simulation results example LS174T (a-e) and SW1222 (f-j) colorectal adenocarcinoma xenografts. (a, f) Three-dimensional visualisations of whole-tumor blood vessel networks, colored according to vessel radius, blood flow and intravascular pressure. (b, g) Three-dimensional rendering of REANIMATE interstitial fluid pressure predictions, in the same tumor as in (a, f), overlaid on the blood vessel network (grey). (c, h) Example *in vivo* measurements of tumor perfusion, acquired in a single slice through the tumor, using arterial spin labelling MRI (left) and the REANIMATE predicted perfusion from a single slice (with vessel structures overlaid in grey). Scatter plots comparing ASL measurements of perfusion and REANIMATE predictions are shown in Supplementary...
Figures 1a and b, respectively. (d, i) and (e, j) show REANIMATE predictions of interstitial fluid pressure and interstitial fluid velocity, respectively, in the same two-dimensional slice as in (c, h).

**Figure 4.** REANIMATE simulations of steady-state fluid dynamics (vascular and interstitial) in an orthotopic murine glioma model (GL261). a) Segmented blood vessel networks, showing tumor vessels (red) and normal brain vessels (blue). The tumor was connected to the brain via several large feeding vessels at the interface between the two tissues. b) The results of REANIMATE vascular simulations, with vessel network color-coded for vessel radius, vascular pressure and blood flow. c) A three-dimensional rendering of REANIMATE interstitial fluid pressure predications, with blood vessel network overlaid. d) Comparison of in vivo perfusion measurements with ASL-MRI and REANIMATE predictions. A scatter plot comparing ASL measurements of perfusion and REANIMATE predictions is shown in Supplementary Figure 1c. A complete slice through the brain is shown for ASL-MRI, with the tumor outlined with a black, dashed line. REANIMATE perfusion predictions show a slice through the tumor. (e) and (f) show REANIMATE predictions for interstitial fluid pressure and interstitial fluid velocity, respectively.

**Figure 5.** REANIMATE simulation of Gd-DTPA (an MRI contrast agent) delivery to an example LS174T tumor, compared with uptake measured in vivo with DCE-MRI. (a) Gd-DTPA enhancement in a slice through the tumor, measured/simulated over 13 minutes. (b) Mean Gd-DTPA concentration as a function of time, for REANIMATE (left) and in vivo data (right). Each curve shows the average uptake at a fractional distance between the perimeter and centre of mass of the tumor. (c) Plot of a line profile through the tumor, at 13 minutes, corresponding to the black lines shown in (a). (d) A histogram of Gd-DTPA concentrations at 13 minutes (also shown as a scatter plot in Supplementary Figure 1d).

**Figure 6.** Dual-fluorophore, optical imaging of the response of colorectal carcinoma models (LS174T and SW1222) to treatment with a vascular disrupting agent (Oxi4503), at baseline and 90 minutes post-dosing. Tumors were injected with lectin labelled with the first fluorophore (AlexaFluor-568) prior to administration of 40 mg kg$^{-1}$ of Oxi4503, to label all blood vessels in the tumors. 60 minutes later, to assess the acute and heterogeneous effects of Oxi4503, a second lectin labelled with a different fluorophore (AlexaFluor-647) was injected, to label vessels that remained perfused. (a) Whole-tumor blood vessel networks, colored according to whether they remained labelled following Oxi4503 (dual-labelled, blue) or were no longer perfused (single-labelled, green). OPT signal intensity images are also...
shown. (b) Box plot showing the distance of single- and dual-labelled vessels from the tumor periphery and (c) the size of single-labelled clusters in both tumor types. (a-c) show that LS174T tumors lost large vascular regions at their centre, whereas SW1222 tumors showed a more distributed pattern of perfusion loss, distributed throughout the tumor. (d) Box plot of blood volume measurements from dual-labelled Oxi4503 and control-treated tumors. Blood volume significantly reduced (p=0.0002, two-sided Wilcoxon rank sum) in both SW1222 and LS174T tumors when treated with Oxi4503, whereas there was no significant difference in control tumors. In box plots, bar-ends define the range of the data, box-ends the interquartile range, and central bars are median values; asterisks denote statistically significant differences (p<0.01, two-sided Wilcoxon rank sum).

**Figure 7.** Results of REANIMATE simulations of blood flow, perfusion and interstitial fluid pressure (IFP), in an LS174T tumor, at baseline (top row) and 90 minutes post-Oxi4503 treatment (middle row). Images showing the change in perfusion *in vivo*, measured with arterial spin labelling MRI, are also shown, alongside histograms of each parameter (bottom row). Small changes in each parameter were observed, which were heterogeneously distributed throughout the tumor, both in simulations and *in vivo*.

**Figure 8.** REANIMATE simulation predictions of Oxi4503 delivery and treatment response. (a) Maps of whole-tumor intravascular and interstitial (tissue) delivery of Oxi4503 from baseline to 90 minutes post-dosing. (b) Box plot of simulated Oxi4503 exposure in branch points connecting single-labelled only, dual-labelled only or a mixture of single- and dual-labelled vessels at 90 minutes post-Oxi4503 delivery. A significantly lower exposure to Oxi4503 was found in single-labelled vessels, as denoted by an asterisk (p=0.004, two-sided Wilcoxon rank sum). Bar-ends define the range of the data, box-ends the inter-quartile range, and central bars are median values. (c-f) A 1 mm-thick slice through an LS174T vessel network, showing (c) the location of dual- and single-labelled vessel segments, (d) simulated Oxi4503 exposure (intravascular and interstitial), (e) intravascular distance from an inlet node and (f) their connectivity score. Yellow arrows show the location of examples of single-label clusters, which are associated with a larger intravascular distance from an inlet, lower node connectivity and mixed (both high and intermediate) Oxi4503 exposure.

**Figure 9.** Connectivity analysis of whole-tumor blood vessel networks. Frequency distributions of a) ln(C) (node connectivity) and b) redundancy distance ratio, r, demonstrating clear distinctions in the distributions for the two colorectal carcinoma xenograft models (SW1222 and LS174T). c,d) Tumor blood vessel networks, with nodes
scaled according to vessel connectivity; the larger the node, the greater the connectivity, for an LS174T tumor (c) and SW1222 tumor (d).
2) *Ex vivo* optical imaging of complete tumor sample

1) *In vivo* tumor model

1a) *In vivo* multimodal imaging

5) Numerical modelling of time-dependent delivery

6) Hypothesis generation and testing

3) Image feature segmentation (vessels)

4) Numerical modelling of steady-state tumor fluid dynamics
Perfusion (in vivo ASL-MRI)

Perfusion (REANIMATE)

Interstitial fluid pressure

Interstitial fluid velocity
Simulated Gd-DTPA concentration (REANIMATE)

0 min  1 min  5 min  8 min  13 min

Measured Gd-DTPA concentration (in vivo DCE-MRI)

0 min  1 min  5 min  8 min  13 min

Mean Gd-DTPA concentration as percentage distance from tumor edge (mM)

[Cd] (mM)

REANIMATE

DCE-MRI

Time (min)

[0-25% 25-50% 50-75% 75-90% 90-100%]

[Cd] (mM)

Edge  Core  Edge

Time (min)

[0.0 0.1 0.2 0.3 0.4 0.5 0.6]

[0.0 0.1 0.2 0.3 0.4 0.5 0.6]

[0.0 0.1 0.2 0.3 0.4 0.5 0.6]
Vessels labelled at baseline only
Vessels labelled at baseline and at 90 minutes

(a) Treatment groups and control: LS174T and SW1222.

(b) Boxplot showing distance from perimeter (µm) for LS174T and SW1222.

(c) Boxplot showing cluster size (number of nodes) for LS174T and SW1222.

(d) Boxplot showing blood volume fraction for LS174T and SW1222.
Simulated blood flow

Baseline

Simulated IFP

mmHg

ml/min/100g

Simulated perfusion

Baseline

90 minutes post-Oxi4503

Measured perfusion (MRI)

Frequency

Interstitial fluid pressure (mm Hg)

Frequency

Perfusion (mL min⁻¹ g⁻¹)

Frequency

Perfusion (mL min⁻¹ g⁻¹)