Chemotherapy Sensitivity of Canine B-Cell Lymphoma

David D Nolte
Honggu Choi

Available at: https://works.bepress.com/ddnolte/27/
Biodynamic digital holography of chemoresistance in a pre-clinical trial of canine B-cell lymphoma

HONGGU CHOI,1 ZHE LI,1 HAO SUN,1 DAN MERRILL,1 JOHN TUREK,2 MICHAEL CHILDRESS,2 AND DAVID NOLTE1,*

1Department of Physics and Astronomy, Purdue University, 525 Northwestern Ave, West Lafayette, IN 47907, USA
2College of Veterinary Medicine Purdue University, 625 Harrison St, West Lafayette, IN 47907, USA
*nlte@purdue.edu

Abstract: Biodynamic digital holography was used to obtain phenotypic profiles of canine non-Hodgkin B-cell lymphoma biopsies treated with standard-of-care chemotherapy. Biodynamic signatures from the living 3D tissues were extracted using fluctuation spectroscopy from intracellular Doppler light scattering in response to the molecular mechanisms of action of therapeutic drugs that modify a range of internal cellular motions. The standard-of-care to treat B-cell lymphoma in both humans and dogs is a combination CHOP therapy that consists of doxorubicin, prednisolone, cyclophosphamide and vincristine. The proportion of dogs experiencing durable cancer remission following CHOP chemotherapy was 68%, with 13 out of 19 dogs responding favorably to therapy and 6 dogs failing to have progression-free survival times greater than 100 days. Biodynamic signatures were found that correlate with inferior survival times, and biomarker selection was optimized to identify specific Doppler signatures related to chemoresistance. A machine learning classifier was constructed based on feature vector correlations and linear separability in high-dimensional feature space. Hold-out validation predicted patient response to therapy with 84% accuracy. These results point to the potential for biodynamic profiling to contribute to personalized medicine by aiding the selection of chemotherapy for cancer patients.

References and links
1. Introduction

Low patient response rates to standard-of-care chemotherapy is a continuing problem in human cancer care. Cancer patients respond positively to therapy in only about 30-40% of cases when averaged over both first-line and salvage therapy. Low response rates present a serious challenge for patient quality of life and longevity. Chemosensitivity tests that rely on two-dimensional cell culture derived from cancer patient biopsies have not achieved significant improvement in the selection of chemotherapy [1]. The two-dimensional assay format loses the important three-dimensional tumor microenvironment that controls many aspects of drug distribution and efficacy [2–5]. Alternatively, tests that rely on xenograft growth in PDX models are time-consuming and expensive [6], and the PDX models produce non-native host microenvironments and possible phenotypic outgrowth that does not represent the personal biology of the cancer patient. Therefore, there is a pressing need to develop a rapid, inexpensive test of efficacy of chemotherapy, applied to \textit{ex vivo} biopsies, that can inform doctors about patient resistance to standard-of-care treatments.

Non-Hodgkin lymphomas (NHL) in dogs have histopathologic, molecular and clinical features similar to NHL in humans [7]. Doxorubicin-based combination chemotherapy is the standard of care for dogs with NHL with the goal to provide durable cancer remission and long-term disease palliation, while preserving quality of life, rather than to cure the cancer. Clinical endpoints can be assessed rapidly in dogs with NHL. An objective response to chemotherapy appears within days following treatment, and the progression-free survival time (PFST) after chemotherapy is approximately 4–9 months [8]. Spontaneous NHL in dogs is biologically and clinically diverse, with response to chemotherapy varying dramatically from dog to dog. The heterogeneity in response to therapy makes NHL in dogs an appropriate model for comparative oncology in which to study the canine analog of the human disease. Despite this heterogeneity, \textit{ex vivo} biodynamic imaging (BDI) has shown great promise as a means to discriminate NHL in dogs that will respond favorably or unfavorably to chemotherapy. For instance, Custead et al. [9] reported that \textit{ex vivo} BDI on canine B-cell lymphoma predicted sensitivity to single-agent doxorubicin chemotherapy with high accuracy.

In this paper, we performed a clinical trial on the application of biodynamic imaging to CHOP standard-of-care combination therapy. The pre-clinical trial enrolled nineteen dogs with multicentric B-cell lymphoma. Biopsies were obtained during initial diagnosis and biodynamic drug-response spectrograms were generated \textit{ex vivo} to the combination therapy and to the individual monotherapies. The spectrograms were converted to feature vectors and
used in the construction of a similarity network and a linear classifier. The performance of the classifier under hold-out validation had an accuracy of 84%.

2. Biodynamic Imaging

Biodynamic imaging [10–12] is an optical imaging technology related to en face OCT [13] using partially-coherent speckle generated by broad-area illumination with coherence detection through digital holography [14–16]. Biodynamic imaging penetrates up to 1 mm into living tissue and returns high-content information in the form of dynamic light scattering across a broad spectral range [17, 18]. The fluctuation frequencies relate to Doppler frequency shifts caused by light scattering from subcellular constituents that are in motion. The speeds of intracellular dynamics range across nearly four orders of magnitude from nanometers per second (cell membrane motion) [19–22] to tens of microns per second (organelles and vesicles) [23–26]. For a near-infrared backscattering geometry these speeds correspond to Doppler frequencies from 0.01 Hz to 50 Hz. Dynamic light scattering in living tissues has been used to identify intracellular transport signatures of diffusive relative to directed motion [27–29], for the detection of apoptosis [30, 31], extracellular restructuring [32] and the response of living tissue to applied drugs and therapeutics such as cytoskeletal drugs [10] environmental drugs [30, 33], Raf inhibitors and metabolic drugs [34], carboplatin and cisplatin [35], and Taxol [36]. None of these previous studies investigated standard-of-care combination therapy in a clinically-relevant disease.

2.1 Optical system

The optical system configuration for biodynamic imaging is illustrated in Fig. 1(a). The low-coherence light passes a beam splitter (BS1) and is divided into the reference and object arms. The object arm collects scattered signal from a specimen through the second beam splitter (BS2). Two lenses form a 4f system (L1 and L2, \( f = 15 \) cm), the last lens (L3, \( f = 5 \) cm) performs a Fourier transformation, and the Fourier image is recorded by the CCD (Qimaging, Rolera EM-C2) at the Fourier plane. The speckle size at the Fourier plane is set to be around 80 \( \mu \text{m} \) which is approximately \( f \lambda / D \) where \( f \) is the focal length of L3, \( \lambda \) is 0.85 \( \mu \text{m} \) and \( D \) (~1 mm) is the width of the stop before L3. Resolution of the reconstructed hologram is dependent on the chip size of the CCD (~8 mm by 8 mm) at the Fourier plane. The low-coherence light source (Superlum, S850-G-I-20) has less than 20 \( \mu \text{m} \) coherence length [34], and coherent interference occurs when the optical path lengths of the object and the reference arm have less than 20 \( \mu \text{m} \) difference for a given scattering depth inside tissue. The advantage of the low-coherence light interferometry is the coherence-gating effect which is selective for scattering signal at a specified depth [10–12]. Scattered signal from tissue is multiply scattered, but by implementing the low-coherence light, three-dimensional volumetric analysis is feasible [12]. The coherence-gate is set to depths around 100 \( \mu \text{m} \) – 300 \( \mu \text{m} \) within the tissue.

Recorded Fourier images at the CCD are analyzed by two-dimensional spatial fast Fourier transform (FFT). Examples of reconstructed mid-sections of a biopsy are shown as an optical-coherence image (OCI) in the first column of Fig. 1(b) before (top) and after (bottom) the application of the dose. These mid-sections are optical sections approximately 10 microns thick, limited by the coherence length of the superluminescent diode. The spatial resolution in these images is approximately 20 microns set by the low-NA optics. The large voxel size contains many scattering elements, enhancing signal-to-noise performance. Higher resolution biodynamic imaging systems have been constructed, but for the large biopsies (approximately 1 mm diameter) used in this study, the larger voxels provide more signal. Fluctuation power spectra are acquired by performing temporal FFTs over reconstructed time series of optical coherence images of dynamic speckle. Examples of motility contrast images (MCI) are shown in the second column of Fig. 1(b) for the respective OCI images. The computations for
the speckle analysis were conducted on Research Computing service systems provided by Purdue University (RCAC). For a single experiment, 16 wells were prepared, and measurements on a single well took 2 minutes with a one-loop data acquisition sequence over the 16 wells taking about 40 minutes. The acquisition sequence was repeated 6 times for the baseline, and 15 times for the post-dose measurement. A single biopsy experiment takes about 14 hours.

![Optical system configuration with low-coherence light (SL) and (b) reconstructed images of B-cell Lymphoma of a canine patient. Optical coherence images (OCI) and corresponding motility contrast images (MCI) are shown for baseline and post-dose measurements.](image)

**Fig. 1.** (a) Optical system configuration with low-coherence light (SL) and (b) reconstructed images of B-cell Lymphoma of a canine patient. Optical coherence images (OCI) and corresponding motility contrast images (MCI) are shown for baseline and post-dose measurements.

### 2.3 Dynamic light scattering

Most intracellular dynamics and flights, especially of macromolecular structures, are driven by active transport through the expenditure of ATP and GTP. Approximately 1% of the energy budget of a cell goes into active transport, primarily driven by molecular motors (kinesin, dynein, myosin, among others) [37], but also by polymerization forces associated with the growth and contraction of microtubules and actin filaments [38]. This intracellular transport is far from thermal equilibrium, and is non-Brownian, yet the statistical properties of active transport are captured as a random walk.

The light-scattering configuration for dynamic light scattering from a moving particle has an initial k-vector \( k_1 \) that is scattered by a small particle into a final k-vector \( k_2 \). The momentum transfer \( q \)-vector in the scattering process is \( q = k_2 - k_1 \), where the magnitude of the transferred momentum is \( |q| = 2k \sin(\theta / 2) \) at the scattering angle \( \theta \). The Doppler angular frequency shift from the central frequency of the incident photon is given by \( \omega_q = q \cdot v = qv \cos \phi = \omega_{q0} \cos \phi \), where \( \phi \) is the angle between the particle velocity and the \( q \)-vector, \( v \) is the velocity of the particle, and \( \omega_{q0} \) is the maximum (or collinear) Doppler angular frequency shift. For backward scattering, the maximum \( q \)-vector for \( \lambda = 840 \) nm in tissue is \( q = 20/\mu \text{m} \). As an example to set the detection scale, the Doppler beat frequency for a particle speed of 1 micron/sec is 3 Hz.

Frequency-domain decomposition of the light fluctuations using tissue dynamics spectroscopy (TDS) [11, 30] produces broad-band fluctuation spectra that contain information on the wide variety of subcellular motions. When pharmaceutical compounds are applied to the tissue, dynamic cellular processes are modified, and these modifications appear as changes in the fluctuation spectra. By applying reference compounds with known mechanisms of action, a library of drug-response spectrograms has been generated against which drug screens may be compared, providing information about the effect of the compound on cellular processes such as necrosis and apoptosis [30]. This type of phenomenological assay is known as a phenotypic profile. Phenotypic profiling has seen a
resurgence in recent years as a systems-based approach to drug discovery and development
[39] that captures the complex interplay of cellular processes affected by the drug candidate.

In dynamic light scattering, coherent speckle is a superposition of the individual partial
waves from the individual scattering sources that are in motion. Because all motions are
averaged isotropically, the mean Doppler frequency shift vanishes. However, the individual
Doppler shifts are preserved in the time-dependent fluctuations in the speckle intensity that
are captured through a field autocorrelation function [40]. The Wiener-Khinchin Theorem
translates between autocorrelation functions and fluctuation power spectra as

$$S(\omega) = FT\{A(\tau)\}$$  \hspace{1cm} (1)

Biodynamic imaging tracks the fluctuation power spectrum in time as $S(\omega,t)$, establishing a
baseline for four hours prior to pipetting the drug into the well. A drug-response spectrogram
is generated as

$$D(\omega,t) = \log S(\omega,t) - \log S_0(\omega)$$  \hspace{1cm} (2)

where

$$S_0(\omega) = \frac{1}{4} \int_0^\infty S(\omega,t) \, dt$$  \hspace{1cm} (3)

is the baseline spectrum. The drug-response spectrogram $D(\omega,t)$ is represented as a time-
frequency fingerprint of the physiological effects of the drug on the living tissue [30].

Averaging the fluctuation spectrum over all intracellular motions produces overlapping
spectra from individual processes. However, fluctuation spectra separate in both frequency
and spectral density, as shown in Fig. 2(a), because the low-frequency spectral weight scales
as $1/\omega_{knee}$ while the Doppler knee frequency scales as $\omega_{knee}$. This fundamental scaling property
of fluctuation spectra separates the processes into broad ranges that are relatively selective in
the fluctuation spectroscopy. It is not possible to image individual motions using biodynamic
imaging, which has tissue-scale resolution, but there is a large body of live-cell imaging
literature that supports a general separation of intracellular processes into broad spectral
ranges. For instance, along the horizontal axis high speeds are organelle and vesicle transport
[23, 25, 26, 41, 42], and low speeds are membrane motions and cellular shape changes [19,
20, 25, 43–46]. Mid-range speeds relate to processes such as nucleus or endoplasmic motions,
membrane undulations and cytoskeletal restructuring. A representative drug-response
spectrogram is shown in Fig. 2(b) for a relapsed B-cell lymphoma biopsy responding to the
anticancer drug doxorubicin. The horizontal axis is in units of intracellular speeds that range
from 3 nm/sec to 3 microns/sec calculated using Eq. (1) to convert measured frequency to
speed. The percent change in spectral content in this figure ranges between $-40\%$ to $+40\%$. 
Fig. 2. (a) A schematic of the spectral contributions from three assumed intracellular processes. The fluctuation spectra separate according to their representative knee frequency, with rheological (cell shape) processes dominating at low frequencies, nuclear and membrane processes at mid frequencies, and organelle dynamics at high frequencies. (b) The data format $D(\omega, t)$ of biodynamic spectrograms illustrated for the case of a B-cell lymphoma biopsy responding to 10 μM doxorubicin. The horizontal axis is the mean intracellular speed obtained from $v = \omega / q$. The vertical axis is time. The sample is dosed after a 4-hour baseline.

3. Methods: canine B-cell lymphoma

Nineteen dogs with histopathologically-confirmed untreated B-cell lymphoma were prospectively enrolled at the Purdue Veterinary Teaching Hospital (PUVTH) between August 2015-December 2016. Only dogs with a multicentric cancer distribution, in which cancerous lesions are most apparent in peripheral lymph nodes, were enrolled in this study. The study protocol was approved by the Purdue Animal Care and Use Committee, and written informed consent was obtained from each dog’s owner prior to enrollment. All dogs underwent surgical lymph node biopsy at the time of enrollment. A portion of each dog’s lymph node biopsy was submitted for histopathologic confirmation of disease, while the residual portion was reserved for ex vivo BDI. Following biopsy, all dogs were treated with a previously-described [8] 25-week CHOP chemotherapy protocol. CHOP combination therapy consists of doxorubicin, prednisolone, vincristine and the active metabolite of cyclophosphamide. The therapy is administered as a combination therapy in humans, but as a sequence of monotherapies in dogs. Objective response to chemotherapy was assessed by caliper-based measurement of peripheral lymph nodes, according to previously-describe criteria [47]. Dogs that completed their 25-week course of chemotherapy were re-evaluated at the PUVTH once monthly until the time of measurable cancer progression or death due to any cause, whichever came first. Progression-free survival (PFS) time for each dog was defined as the time (in days) from initiation of CHOP chemotherapy to the time that the sum of the longest diameters of up to 5 peripheral lymph nodes was at least 20% greater than its lowest recorded value. Progression-free-survival times are shown in Fig. 3 for the 19 canine patients enrolled in this study. All dogs experienced measurable disease progression prior to death. There are approximately three groups: dogs with PFS less than 50 days, dogs with PFS between 50 and 100 days, and dogs with PFS greater than 200 days. These three groups are characterized as having short, medium and long PFS times. In much of the following analysis, the short and medium PFS times are pooled into a short-PFS group, characterized as resistant to CHOP therapy, contrasted with the long-PFS group that is characterized as sensitive to CHOP therapy.
Fig. 3. Progression-free survival (PFS) for 19 dogs treated with CHOP. The last two dogs had PFS times greater than 365 days.

For biodynamic imaging, needle core biopsies or excised lymph nodes were collected into chilled RPMI-1640 cell culture medium and minced into 1 mm³ samples and placed in 96 well plates. A thin layer of low-gel temperature agarose (Sigma-Aldrich) was used to immobilize the samples, and all samples were then overlaid with RPMI-1640 medium containing 10% fetal bovine serum (Atlanta Biologicals), 100 U/mL penicillin, 0.1 mg/mL and 25 mM HEPES.

The active metabolite of cyclophosphamide (4-hydroxycyclophosphamide) was purchased from Niomech (Bielefeld, Germany). All other drugs were purchased from Selleck Chemicals (Huston, TX). Drug stock solutions were prepared using DMSO. The drug concentrations were chosen based upon levels within the range that are clinically achievable in vivo. The concentration of the drugs tested in complete RPMI-1640 medium were doxorubicin (10 μM), the active metabolite of cyclophosphamide (4-hydroxycyclophosphamide 5 μM), vincristine (60 nM), and prednisolone (0.6 μM). Medium containing 0.1% DMSO was used as the negative control.

4. Biodynamic profiling of canine B-cell lymphoma

Biodynamic time–frequency spectrograms $D(\omega, t)$ were acquired for the 19 enrolled dogs under 5 treatments (CHOP, doxorubicin, prednisolone, vincristine and cyclophosphamide) as well as wells dosed only with the DMSO carrier as negative controls. Each treatment was measured using three to five replicates in randomized well locations. The averaged spectrograms are shown in Fig. 4 for raw average spectrograms and DMSO-subtracted average spectrograms. The negative control (DMSO) displays a broad inhibition of cellular activity, possibly representing the response of the underlying biopsy material to the trauma of surgery and sample dissection. The non-zero baseline of each spectrogram likewise reflects the changing character of the biopsy sample. The negative control displays the background response for all biopsy samples. When this is subtracted from the drug responses, characteristic signatures emerge. For instance, both CHOP and doxorubicin elicit a response with enhanced low and high frequencies and suppressed mid frequencies. This biodynamic spectrogram pattern has been correlated previously with early-stage apoptosis [30].
vincristine and cyclophosphamide elicit general enhanced motions, especially in the mid-frequency range, while prednisolone produces a limited response, perhaps because it is a steroid that is used as part of the CHOP combination for its immunological effects which are largely absent from the tumor biopsy tissues.

![Fig. 4. Raw average spectrograms (top row) and DMSO-subtracted average spectrograms (bottom row). Frequency spans from 0.01 to 10 Hz on the horizontal frequency axis, and from −4 hours to 12 hours on the vertical time axis. The treatment is applied at $t = 0$ (horizontal line on each spectrogram). The color range is from −70% (blue) to + 70% (red).](image)

The spectrograms were averaged into two groups, a group with long PFS (sensitive) and a second group with medium and short PFS (resistant). The average spectrograms are shown in Figs. 5(a) and (b) for the two groups. The long-PFS phenotype is on the bottom row of spectrograms. There are significant and important differences, representing the biodynamic phenotypes for short and long PFS canine B-cell lymphoma patients. The long-PFS spectrograms for CHOP and doxorubicin show classic signatures of early-stage apoptosis [30] with enhanced low and high frequencies and suppressed middle frequencies. Prednisolone and cyclophosphamide display blue shifts (enhanced high frequencies and suppressed low frequencies), while vincristine displays an enhanced middle frequency. In contrast, the short-PFS phenotypes show very different signatures. CHOP displays broad mid-frequency enhancement, and doxorubicin displays a strong blue shift, while prednisolone, vincristine and cyclophosphamide display strong mid-frequency enhancements. The differences between the short and long PFS phenotypes are shown in Fig. 5(c). The short-PFS doxorubicin response has enhancements at high frequency (blue shift) relative to the long-PFS response. In addition, the overall short-PFS phenotype has enhanced mid frequencies relative to long-PFS across all treatments, which has been observed previously for canine B-cell lymphoma response to doxorubicin [9]. In terms of Doppler light scattering, these data show a clear and consistent trend for overall enhanced spectral responses in short-PFS phenotypes relative to long-PFS phenotypes when under treatment.
Fig. 5. Average spectrograms for (a) the resistant and (b) the sensitive subsets of the canine patient cohort. A strong phenotypic difference exists for nearly all drugs. The sensitive phenotype displays early apoptotic character (which is a desired chemotherapy outcome) for CHOP, doxorubicin and prednisolone. The responses to vincristine and cyclophosphamide show strong spectral enhancements for the resistant patients. (c) Difference spectrograms for short-PFS relative to long-PFS patient responses. There is overall enhanced higher frequency content in all cases, and a blue-shift of doxorubicin for short-PFS relative to long-PFS.

5. Classification analysis

A key goal of this preclinical trial is to construct a chemoresistance classifier that takes a set of treatment spectrograms for a single patient and predicts whether the patient will have a short or a long PFS under that selected treatment regimen. The construction of the classification algorithm is based on simple linear separability in a medium-dimensional feature space. The time-frequency drug-response spectrograms for each patient are deconstructed into a set of so-called features, each capturing either local or global spectrogram patterns. Examples include: overall enhancement/suppression over all frequencies (ALLF, ALLFT); localized low, mid and high (HI) frequencies; red shifts or blue shifts (SDIP); and different time dependences in response to the applied treatment (SDIP vs. SDIP2 and ALLF vs. ALLFT). A description of many of these feature vectors can be found in previous publications on biodynamic imaging [30, 35, 48]. Each tested drug (CHOP, Doxorubicin, Prednisolone, Vincristine and Cyclophosphamide) has a set of these biodynamic biomarkers.

5.1 Linear separability analysis

Linear separability analysis seeks to find a (N-1)-Dim hyperplane that best separates the short-PFS and long-PFS points in the N-Dim feature space. An efficient method to find this hyperplane is the algorithm known as a neural perceptron. A perceptron is the simplest possible neural net with a single neuron having multiple inputs (the values of the feature vector) and a single output (clinical outcome). The optimization process finds the neural weights of the inputs and the bias of the neuron that minimize a cost function. The neuron sigmoidal response function is assumed to be a hyperbolic tangent (tanh) that saturates to ±1 for large positive/negative arguments that are compared to the ±1 classification based on the PFS. The cost function uses a chi-squared error and a regularization cost. The regularization cost measures the mean squared distance from the mean hyperplane that is defined as the
bisector of the vector between the center of mass of each class of points. Regularization is required when finding hyperplanes in order to keep the hyperplane parameters from drifting to large values during optimization. The regularization factor in our analysis was chosen to be 10%, which is a commonly-used rule-of-thumb [49]. The perceptron is trained using gradient descent on the cost function.

Hyperplanes that separate data sets are relatively ubiquitous in high dimensions and can be dominated by a noisy channel that carries little signal. This is the classic over-fitting problem in which a classifier becomes merely a look-up table for the training set. To guard against over fitting, standard hold-out validation was used [49]. In the hold-out procedure, the perceptron algorithm is trained on all but one patient that is held out from the analysis. Once the algorithm is trained, the held-out patient is tested and compared against the known clinical outcomes. This process is then repeated, retraining the algorithm each time, for each patient. The hold-out process causes the hyperplane to vary when proceeding from patient to patient. However, the hyperplane parameters (weights on the biomarkers) are monitored and only biomarkers that have consistent weights through this process are used for final classification and for the similarity analysis (Section 5.2). The random character of the stochastic optimization process can have different final states of the optimization. The average of 4 independent runs of the perceptron are shown in Fig. 6 as the quorum value. Of the 19 dogs, three have false reads: (TP = 5, TN = 11, FN = 1, FP = 2) for an assay accuracy of 84%. In a two-sample unpaired t-test performed on the two-class classifications, the p-value was less than 0.05, and the mean separation was 0.62 with a confidence interval of CI = [0.2 - 1.0].

An alternative linear separability analysis was performed based on log likelihood estimation [49] during hold-out. The values of each biomarker are histogrammed into resistant \( P_R(v_a) \) and sensitive \( P_S(v_a) \) probability distribution functions (PDF) according to their clinical outcomes. The feature value of the held-out dog is then compared to each PDF. The log likelihood that a held-out dog belongs to the resistant or sensitive class is then

\[
LL = \sum \log \frac{1 + P_R(v_a)}{1 + P_S(v_a)}
\]

where the sum is over all biomarkers. This approach has the advantage that no thresholds are set while biomarkers that carry little information contribute almost nothing to the log likelihood. The log likelihood for each of the held-out dogs is shown in Fig. 6. They match closely with the perceptron quorum.
5.2 Similarity analysis

The biomarker features that carry the most information were selected during the hold-out validation procedure by identifying the biomarkers that are most consistently selected during the hold-out process. It is necessary to select biomarkers from inside the hold-out process to avoid selecting biomarkers that merely correlate with patient outcome. There were nine biomarkers that consistently separated the dogs into short and long PFS during the hold-out procedure. An example of a feature vector matrix is shown in Fig. 7(a) for the nine features across the 19 canine patients. Note that only the drugs CHOP, doxorubicin and cyclophosphamide are represented in this set. Vincristine and prednisolone were found not to differentiate between the short- and long-PFS dogs. The matrix of feature vectors is grouped into resistant (short-PFS) and sensitive (long-PFS) groups. Each row is the feature vector for that patient and defines a point in a 9D (9-dimensional) feature space associated with that patient.

The similarity analysis constructs a similarity matrix that relates the pair-wise similarity between each patient. There are several possible measures of similarity. We chose to use the direction cosine between two feature vectors as the measure of similarity. The direction cosine is insensitive to overall magnitudes and measures only whether vectors are pointing in similar directions in the feature space. This measure is relatively robust against the patient-to-patient biopsy variability that produces a high variance among the individual elements of the feature vectors. The similarity matrix for the enrolled dogs is shown in Fig. 7(b). The order of the patients has been put into groups as determined by the perceptron analysis. The matrix is approximately block-diagonal, with two main red blocks and mainly blue off-diagonal blocks. The blocks relate to the short- and long-PFS groups, except for the three dogs Cli, Jo and Jul that were false reads.
Fig. 7. (a) Feature vector matrix for nineteen patients and 9 features (N-Dim = 9) grouped into resistant (short-PFS) and sensitive (long-PFS) groups. The patient sorting order is based on PFS times (see Fig. 3). (b) Similarity matrix for the nineteen patients. Each matrix element is the direction cosine between the two feature vectors. The short-PFS and long-PFS groups cluster into block-diagonal (except for Jul). The color scale is from $-1$ (blue) to $+1$ (red).

The similarity matrix is used as the input to construct a similarity network, shown in Fig. 8. The off-diagonal elements of the matrix are compared to a threshold. If the direction cosine magnitude is larger than the threshold (in this case 0.3), a link is drawn between those two patients represented by nodes in the network. The nodes are color coded into short-PFS (blue) and long-PFS (red). The short-PFS patients cluster tightly, except for Jul, who clusters strongly with the long-PFS group. Two dogs with long-PFS (Jo and Cli) cluster with the short-PFS dogs. The similarity network shows a separate clustering of the two phenotypes with several marginal cases. Many of the long-PFS patients cluster into a subcluster with an intra-class link probability of 33%, while the subcluster containing the short-PFS patients had a larger intra-class link probability of 50% for the similarity threshold selected here. This indicates that the resistant patient profiles are more similar to each other than the sensitive profiles are to each other. The patient nodes with the highest degrees corresponded with the strongest and most consistent classifier results from the perceptron analysis.

6. Discussion and conclusion

This article describes the first application of biodynamic digital holography to assess the response of ex vivo living biopsies to a standard-of-care combination therapy (CHOP) that is prescribed for both human and canine patients with non-Hodgkin’s B-cell lymphoma. In the ex vivo testing, separate biopsy samples were exposed to physiologically-relevant doses of each of the single-agent therapies (doxorubicin, cyclophosphamide, prednisolone and vincristine) as well as the combination therapy. By testing the single-agent therapies, the drug-response biodynamic signatures of each agent (having distinct mechanisms of action) are isolated, allowing the individual responses to be assessed in addition to response to the combination therapy. The mean-subtracted and normalized biomarkers are feature vectors in a high-dimensional feature space representing the response of each of the 19 canine patients to the ex vivo treatments. The analysis identifies biodynamic biomarkers, selected from the collection of single-agent and combination therapies, that are most consistent in the classification of patient response to therapy.
Fig. 8. Similarity network with links that reflect similarities between dogs. The nodes are colored blue (short-PFS) and red (long-PFS). Links are shown for direction cosines greater than 0.3. Nodes representing the average long and short PFS responses are included. Two false positives (Cli and Jo) and one false negative (Jul) are clearly clustered with the opposite groups.

Relative similarities among all pairs of patients are calculated using the feature vectors. While there are many ways to define similarity in a vector space (e.g., Euclidean distance, Manhattan distance, Kolmogorov-Smirnov test, Riemannian metrics, correlation, covariance, direction cosine, contrast, among others) we found that the direction cosine provides a simple, robust and reproducible measure of similarity among the patient drug-response feature vectors. The direction cosine is independent of overall magnitude of the feature vector, while capturing the similarities in the patterns of feature values. The resulting similarity matrix is converted into a similarity network by selecting a threshold that converts the similarity matrix into an adjacency matrix for the network. The key result of the similarity network analysis is the segregation of the long-PFS and short-PFS patients into two relatively distinct subclusters.

The classifier results were obtained through training a simple neural perceptron. The perceptron was trained using the hold-out procedure in which 18 of the dogs were used for training and the 19th dog was classified, and then repeating the hold-out procedure 19 times. The perceptron identified the best linear separability of the point cloud in the feature space separating the sensitive from the resistant patients. All but three patients (Cli, Jo and Jul) were correctly classified for the majority of the training runs using gradient descent for cost-function minimization. Three additional patients were marginal (Yum, Cod and Bai). The patient Jul showed very strong biodynamic signatures of therapy sensitivity, yet had a medium PFS time similar to Boo and should have been classified as resistant. In the clinical assessment, Jul was a very large dog and subsequently may have been under-dosed. The selection of individual doses is based on patient surface area, which is a procedure that may under-dose large patients. The fact that Jul was an outlier in the patient size distribution may account for its incorrect classification in this trial.

On the other hand, no distinct clinical observations could be highlighted for the other two misclassified patients and the three marginal cases. It is important to point out that the biodynamic assay measures the response of biopsy tissues ex vivo and disconnected to the immune system of the patient, while the ability to sustain remission depends on an individual’s immune system. Measures of endogenous anti-tumor immunity in lymphoma patients correlates with survival, and it is becoming clear that the immune system is probably
a major factor keeping a cancer in remission once that remission has been produced by the chemotherapy. Furthermore, there may be some physiologic processes within the patients that the biodynamic profile cannot capture, such as drug delivery to the tumor. Finally, tumor heterogeneity as well as biopsy subsampling heterogeneity contributes large variances to the biodynamic measurements with only 3 to 5 replicates for each treatment. The multiple subsamples help to average over the variability, but some patients may still have large variances. Future developments of higher multiplexed assays and stronger histological guidance for subsample dissection may further reduce the effects of sample variance. Producing spectrograms from smaller regions could image this heterogeneity directly, further improving spatial selection of the most relevant drug responses. In addition, it would be interesting in future work to study whether patients displaying marginal biodynamic response signatures have different genotypes.

The biodynamic signatures of drug mechanisms of action are only beginning to be mapped out. The connection between physiological effects of a drug, and especially the personal response, are still largely unknown. Nonetheless, the high accuracy of this preclinical trial and the strong phenotypic differences between sensitive and resistant patient responses provide a significant approach for predicting patient response to therapy. Future development of biodynamic profiling will continue to utilize tool compounds and reference drugs with known mechanisms of action to identify the biodynamic signatures of specific subcellular processes. More extensive use of neural networks in the analysis of biodynamic biomarkers may help in this process as well as in the selection of the biomarkers that carry the most information related to drug sensitivity. An important next step in the analysis, using multilayer neural networks with hidden-layer neurons, is to develop a continuous-valued predictor that can differentiate strong responders from moderate responders, providing a continuous degree of sensitivity to help clinicians make decisions. Future developments of biodynamic profiling will seek to identify which therapies are best matched to individual patients for personalized therapy selection. Additional biodynamic therapy assessment studies are currently underway in human ovarian and breast cancer as well as human esophageal cancer.

Funding

National Science Foundation (NSF) (1263753-CBET); National Institutes of Health (NIH) (RO1-EB016582). D. Nolte and J. Turek have a financial interest in Animated Dynamics, Inc. which has licensed biodynamic technology from the Office of Technology Commercialization at Purdue University.

Disclosures

The authors declare that there are no conflicts of interest related to this article.