internalisation, whereas multiple monoclonal antibodies lead to efficient internationalisation by formation of an antigen–antibody lattice at the cell surface.

An example was shown that involved combining two monoclonal antibodies, trastuzumab and pertuzumab, which do not compete for the same HER-2 site. The combination was much more effective than either used alone.

DEVELOPMENT OF RESISTANCE TO ANTI-ERBB RECEPTOR ANTIBODIES: Only a third of breast cancer patients with Her-2 overexpressing tumours respond initially to trastuzumab, implying that most patients have primary resistance. Also the majority of responding patients demonstrate disease progression after months of treatment initiation because they develop secondary resistance. Nagata et al. described the development of resistance to monoclonal antibodies via deficiency of PTEN, a dual phosphatase that mainly dephosphorylates position D3 of membrane phosphatidylinositol-3,4,5 trisphosphate. PTEN-deficient breast cancer patients had significantly poorer responses to trastuzumab-based therapy than those with normal PTEN levels. The authors concluded that PTEN deficiency predicted resistance to trastuzumab and that PI3K inhibitors rescued PTEN loss-induced trastuzumab resistance in a model system, suggesting PI3K-targeting therapies as a means of overcoming acquired resistance.[4]

Another possible mechanism for resistance to monoclonal antibodies against tyrosine kinase receptors was described by Nagy et al. who claimed that MUC4, a membrane-associated glycoprotein that inhibits trastuzumab binding to Her-2, was higher in a trastuzumab-resistant cell line than in sensitive cell lines.[5] Levels of MUC4 were inversely correlated with the trastuzumab-binding capacity of single cells. Knockdown of MUC4 expression by RNA interference increased binding. It is postulated that masking of ErbB2 by MUC4 is a mechanism for trastuzumab resistance. In addition resistance may occur via insulin-like growth factor-I (IGF-I) receptor interactions, as described by Nahta and colleagues.[6] They found that Her-2 interacts in a unique way with IGF-I receptor in trastuzumab-resistant cells possibly enabling cross talk between IGF-I receptor and Her-2. The IGF-I receptor/Her-2 heterodimer could contribute to trastuzumab resistance and serve as a potential therapeutic target in breast cancer patients whose disease has progressed while on trastuzumab.[5]

In conclusion, defective immune responses (e.g. Fc receptors) might underlie primary resistance to trastuzumab, but acquired (secondary) resistance seems to involve compensatory signalling mechanisms.

Various reports have addressed the issue of resistance to tyrosine kinase inhibitors (TKI). Despite significant responses to gefitinib (Iressa®), most patients with non-small-cell lung cancer (NSCLC) relapse after 6–18 months. Kobayashi et al. described the case of a patient with *EGFR*-mutant, gefitinib-responsive, advanced NSCLC who relapsed after 2 years of complete remission. Secondary resistance developed via a second point mutation in EGFR.[7] This phenomenon might be responsible for germ-line mutations in families with multiple cases of NSCLC. It is not known whether this mutation exists independent of gefitinib exposure. Acquired resistance to lapatinib was associated with up-regulation of oestrogen receptor signalling.[8] These findings appear to provide a rationale for preventing the development of acquired resistance by simultaneously inhibiting both oestrogen receptor and ErbB2 signalling pathways.

In summary tumours demonstrate remarkable plasticity, seeming to switch pathways when one is effectively inhibited. Clarifying the mechanisms of drug action is critical for understanding resistance to targeted therapies. Resistance mechanisms richly harness compensatory pathways (PI3K/Akt, Her-3 and IGF-I receptor). Understanding drug resistance will likely identify novel drug combinations and elucidate the mechanisms of tumour cell escape, which must involve the ability to up-regulate certain pathways.

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APPLICATION OF NOVEL IMAGING TECHNIQUES FOR EARLY CLINICAL TRIALS

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In future, proteomics biomarkers may be used involving a novel technique for studying protein–protein interactions, and high-throughput and high-content imaging methods. Light, being of zero mass and unmatched in speed, is an efficient means of sending information to and from structures and materials. Therefore, optical imaging methods, with the aid of suitable contrast-generation biomarkers, are appropriate to conveying a great deal of information about tumours cells and tissues, and their response to targeted therapies.

A recently formed Optical Proteomics Consortium (http://www.opticalproteomics.org) is a research network dedicated to developing new optical technologies for monitoring protein interactions and post-translational modifications in cells and tissues. Its goal is to advance understanding of the function of proteins and to determine protein interactions and interaction networks *in situ*. It aims to develop techniques that are complementary to established, but more indirect methods (e.g. matrix-assisted desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry) that enable the analysis and identification of very small amounts of protein. In contrast, optical techniques are *in situ* methods which perform the analyses in a physiological context.

ROLE OF PROTEOMICS AND FUNCTIONAL GENOMICS IN BREAST CANCER: Breast cancer treatment involves surgical excision, cytotoxic chemotherapy, radiotherapy, hormonal manipulation and targeted biologic agents. Breast cancer patients face a great diversity of possible clinical outcomes and vary widely in the benefits, risks and toxicities deemed acceptable. Current prognostic features estimate the risk of recurrence for groups only; ideally, however, we would like to be able to identify genetic and proteomic profiles that could predict aggressive behaviour of breast cancer cells in individuals and be associated with specific outcomes.

Despite the availability of established methods for genomic profiling via gene expression arrays, there is limited evidence which suggests that treatment decisions based on such profiles save lives. The specificity of therapeutic agents is determined by their effect on the proteome, involving multiple proteins and protein networks. Protein function depends on post-translational modification, glycosylation, phosphorylation and so forth. One of the goals of a current proteomic study is to process as many archived cancer tissues as possible in an attempt to correlate tissue microarray (TMA) results with clinical outcomes. If a link is found between one or more markers and clinical outcomes, prospective studies could be carried out.

Fluorescence lifetime imaging (FLIM)

Studies are ongoing that investigate whether a strong interaction between PKC-α and ezrin in breast cancer tissue confers on a tumour an increased ability to survive and metastasise. PKC-α is a signalling protein that opposes apoptosis and is implicated in tumour progression. As previously shown,[1] using *in vitro* systems, ezrin is a protein that provides a direct link between the cytoskeleton and the plasma membrane. An extension of this method involves fluorescent-labelled antibodies applied to tumour microarrays (TMAs), which are then analysed using fluorescence lifetime imaging (FLIM). Interactions are quantified using Förster resonant energy transfer (FRET), a very powerful tool capable of quantifying protein interactions.[2] FRET relies on the presence of two fluorescent tags, a donor and an acceptor molecule. It requires labelling of target proteins with specific fluorescent tags, via either genetic encoding or chemical means. When the donor molecule is excited with a short light pulse of appropriate wavelength, its fluorescence emission profile is modified by the presence, or otherwise, of the acceptor molecule. When energy is transferred from a donor molecule to the acceptor, the donor emission is quenched and this is reflected in the reduction of the donor fluorescence lifetime: the interaction causes energy to be transferred from the donor fluorophores to acceptor fluorophores, producing increased emission from the acceptor and a decreased fluorescence lifetime in the donor.[3] A histogram of the arrival times of photons from the donor can then be compiled. The lifetime is defined as the average amount of time a molecule spends in an excited state following absorption of light. Fluorescence lifetimes range from pico- to nanoseconds and, in this instance, indicate how close the two proteins are; the relative amplitudes of the quenched and unquenched lifetimes give a measure of how many donor–acceptor pairs are present. FRET only occurs when the two spectrally overlapping fluorophores are in close proximity to one another (typically < 10 nm). Although such techniques are well established,[4–8] for studying cellular systems *in vitro*, the technological challenge is to scale them up to allow quantification of significant imaged areas, involving a large number of cells. This technique was used on breast tissue TMA samples to study differences in protein interaction in basal cell invasive cancer. For example, in normal basal duct epithelium, no FRET occurs; that is, ezrin and PKC-α do not interact. In grade 2 invasive ductal breast cancer, however, such an interaction does occur and FRET is present. This study was performed using antibodies to ezrin (2H3, Cy2-labelled) and to PKC-α (T(P)250, Cy3-labelled) and was based on an 84 section TMA per slide. Currently, lifetime distributions give a clear distinction between FRET and control sections at around 1.5 and 1.8 ns average lifetimes, respectively. A range of software tools is employed to provide detailed kinetic analyses and results where the distribution of errors is understood. In particular, the use of 'global' fitting algorithms allows a greater 'contrast' to be obtained between control (*ca.* 1.8 ns) and FRET-ing areas (exhibiting mixtures of 1.8 ns and *ca.* 500 ps).[9] Nevertheless, to make such a study most effective, tissue areas that are not contributing to the signal would ideally be excluded by software tools that highlight, either manually or by automated means, specific areas of interest. Such approaches minimise the contributions from, e.g. autofluroscence emissions, and thus provide a more valid contribution from the proportions of tissues exhibiting FRET. Manual delineation of specific areas inevitably introduces an operator-dependent bias, as well as slowing down analyses and to this end, various automated methods, based on techniques used in pathology to grade tissue sections are under development. In particular, the texture of the imaged sample gives a great deal of information and Prof. Vojnovic described a method for automated texture analysis that classifies images using a range of measures. He showed an example in which the imaging system 'looks' at extreme cases of distinct textures, aided initially by a trained observer, then uses a range of measures classified in a multidimensional vector space and analyses them to identify specific regions of interest (e.g. membrane-bound staining, regions of invasive tumour edge, cytoplasmic areas, etc.) to determine separate FRET patterns within these.

Further developments in instrumentation to acquire data from a complete TMA are necessary. Conventional microscopy methods must be significantly adapted to acquire, in an unsupervised manner, images from the complete array, as is required to preserve spatial information and relationships. Large numbers of images are eventually 'stitched' together to provide a complete image. Continuous automatic focusing methods are essential to preserve sub-cellular resolutions. Although the work is currently slow, results are very precise and accurate.

THE FUTURE: Currently, we are moving away from normal microscopy platforms for this type of work. Under development is a prototype imaging station dedicated to high-throughput work. This arrangement eliminates traditional approaches and is equally suited to acquiring data from other 'large-area' samples. An obvious example is the use of high-density multiwell plates for assays involving drug action. One of the current rate-limiting steps is the use of scanned optical beams and single-point detection.[10–13] Highly parallel time-resolved detectors are however under development and the future of this technique will be dependant on complementary highly parallel signal processing instrumentation. Furthermore, data handling and analysis requires multiprocessor computer architectures and a high level of automation.

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HOW CAN PROTEOMICS HELP IN THE DEVELOPMENT OF MOLECULARLY TARGETED THERAPY OF CANCER?

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Matrix-assisted laser desorption/ionisation (MALDI) is a 'soft' ionisation technique that most often uses a time-of-flight (TOF) mass spectrometer. The advantage of MALDI-TOF TOF mass spectrometry (MS) over alternative approaches are ease of use, simple sample preparation and high throughput, rendering it an ideal tool for large scale clinical application.[1] Using MALDI-TOF MS for clinical specimens can yield important information about disease state, drug response and/or efficacy, and drug toxicities. It can also be used to analyse biomolecules that could serve as classifiers to determine which patients will most likely respond to certain molecularly targeted therapies. The use of MALDI-TOF MS as a clinical tool has been overshadowed by some notable failures. Focusing on reproducibility and validation, we have shown that MALDI-TOF MS can be successfully applied to protein profiling in a clinical setting.

MASS SPECTROSCOPY AS A DIAGNOSTICS TOOL: Achieving sufficient reproducibility in mass spectrometry for use in a clinical setting is hardly a trivial issue. Mass spectra can be highly var-