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REPORT

A global view of protein expression in human cells, tissues, and organs

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Defining the protein profiles of tissues and organs is critical to understanding the unique characteristics of the various cell types in the human body. In this study, we report on an anatomically comprehensive analysis of 4842 protein profiles in 48 human tissues and 45 human cell lines. A detailed analysis of over 2 million manually annotated, high-resolution, immunohistochemistry-based images showed a high fraction (>65%) of expressed proteins in most cells and tissues, with very few proteins (<2%) detected in any single cell type. Similarly, confocal microscopy in three human cell lines detected expression of more than 70% of the analyzed proteins. Despite this ubiquitous expression, hierarchical clustering analysis, based on global protein expression patterns, shows that the analyzed cells can be still subdivided into groups according to the current concepts of histology and cellular differentiation. This study suggests that tissue specificity is achieved by precise regulation of protein levels in space and time, and that different tissues in the body acquire their unique characteristics by controlling not which proteins are expressed but how much of each is produced.
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Introduction

Recently, a detailed study of 1% of the human genome showed that chromosomes are pervasively transcribed and that the majority of all bases are included in primary transcripts (Birney *et al*, 2007). This has been recently confirmed by extensive parallel sequencing of transcripts, which has shown that a large fraction (70%) of the predicted 20 400 (Clamp *et al*, 2007) protein-encoded genes can be detected in a single human cell line (Sultan *et al*, 2008). In addition, a vast number of alternative splicing events have been identified, adding to the complexity and ubiquitous expression of the human transcriptome (Tress *et al*, 2007; Wang *et al*, 2008). This plasticity at the RNA level is even further accentuated by the presence of an immense numbers of inhibitory RNAs (Yelin *et al*, 2003; Katayama *et al*, 2005) and the recent discovery that tens of thousands of binding sites are present across the genome, as shown by genome-wide profiles of the DNA binding of mammalian transcription factors (Robertson *et al*, 2007). The question arises whether this ubiquitous RNA expression is also translated to the protein level and how this relates to central biological questions regarding the link between protein expression profiles and cellular phenotypes, and the divergence of protein levels in differentiated cells from normal and disease tissues.

We have previously described the high-throughput generation of antibodies and subsequent creation of a Human Protein Atlas (http://www.proteinatlas.org) based on tissue microarrays (TMAs), immunohistochemistry, and immunofluorescence (Berglund *et al*, 2008). In this study, we describe, for the first time, a systematic analysis of global protein expression patterns generated from this public resource. We have

examined the spatial distribution and the relative abundance of proteins in the different cell populations of various tissues in all major human tissues and organs, including the brain, liver, kidney, lymphoid tissues, heart, lung, skin, GI tract, pancreas, endocrine tissues, and the reproductive organs. Thus, it has been possible to assess the functional associations between phenotypically different cells and to study the relationship between protein expression profiles and developmental origin.

Results and discussion

Global protein profiling in 65 normal human cell types

An unsupervised cluster analysis (Eisen *et al*, 1998) was carried out based on the protein levels in 65 normal cell types. The variability introduced by the individual experimental staining protocol, including the choice of antibody dilution and antigen retrieval methods, was addressed by the use of TMAs (Kononen *et al*, 1998), thus allowing parallel determination of the relative levels of a particular protein target, within its dynamic range, across hundreds of biosamples (Warford *et al*, 2004; Taylor and Levenson, 2006). Annotations of more than 2 million images were performed by certified pathologists, and the relative expression level of a particular protein was translated into a four-color code ranging from strong (red), moderate (orange), weak (yellow), and no (white) protein expression (Kampf *et al*, 2004; Bjorling *et al*, 2008). It is important to point out that this color code represents the relative expression levels of a particular protein across tissues and organs, but the absolute levels of each protein have not been determined and could vary by many orders of magnitude. Although the level at which it is appropriate to divide cell types into categories is arbitrary, the resulting heat map (Figure 1) shows that the cells cluster into groups that could be expected on the basis of traditional embryology, histology, and anatomy, with most of the cells divided into six major groups: (i) cells of the central nervous system (CNS); (ii) hematopoietic cells; (iii) mesenchymal cells; (iv) cells with squamous differentiation; (v) endocrine cells; and (vi) glandular and transitional epithelial cells. Further subdivision is also evident, as exemplified by (i) separate subgroups containing neuronal and glial cells in the CNS cluster; (ii) subdivision of cells from the male and female genital tracts; and (iii) a distinct subcluster of glandular cells from the GI tract. The liver hepatocytes, together with striated and heart muscle cells (myocytes), have the most divergent protein profiles.

Sensitivity analyses were also carried out using proteins encoded from single human chromosomes to obtain a random stratification of a substantially smaller subset of the proteome. Similar dendrograms were obtained for the chromosome specificity (Supplementary Figures S1–S3), as well as random groups of 200 antibodies (Supplementary Figures S4–S6), suggesting that the phenotype of the cells is generated by a large fraction of human proteins, as a random sampling of only ~1% of the protein-encoded genes (200 proteins) are sufficient to group the cells in a nearly identical pattern compared with the whole data set. The dendrogram shows that cells with similar cellular functions exhibit similar protein profiles, as exemplified within the hematopoietic cell cluster, in which germinal center cells and peri-follicular lymphoid cells have a more closely related expression profile than the more distant hematopoietic cells in the bone marrow. Similarly, the myocytes in cardiac and striated muscle have similar expression profiles, and these are distinctly different from smooth muscle cells and other stroma cells in the mesenchymal cell cluster.

Protein profiles and developmental origin of the cells

The similarity in protein profiles often coincides with the putative developmental origin (endoderm, ectoderm, or mesoderm) of cell types, as shown by different color codes for branches of the dendrogram (Figure 1). This can be exemplified by glandular cells in the GI tract, which are derived from the endoderm, cells in the CNS originating from the neuro-ectoderm, and hematopoietic/mesenchymal cells derived from the mesoderm. For certain cell types, morphological differentiation supersedes developmental origin, as exemplified in the cluster of cells with squamous differentiation, in which cells from all three germ layers are represented: surface epithelia of the esophagus (endoderm), epidermal cells from the skin (ectoderm), and surface epithelia from intravaginal elements of the cervix (mesoderm). These patterns show that global protein profiles in differentiated normal cells reflect the pluripotent origin of the corresponding stem cells in different germ layers, but that functional convergence also exists resulting in similar expression profiles that are independent of developmental origin.

The tissue-specific protein expression in 65 cell types corresponding to 48 tissues and organs

Expression analysis can be used to estimate the relative level of different protein expressions in each cell type. An analysis of the fraction of cell types containing each protein is shown in Figure 2A, with the cells classified into groups exhibiting strong (red), medium (orange), or weak (yellow) expression. The analysis indicates that a large proportion of the proteome is expressed across many of the 65 cell types: 20% (949) of the proteins are found at detectable levels in $\geqslant$60 cell types, whereas only 3% (150) are detected in less than six cell types. A similar analysis was conducted to study the fraction of analyzed proteins (4842) that are detected in each cell type (Figure 2B). This showed that a large fraction of protein-encoding genes are present in any given cell type, with an average of 68% (range 40–84%) of all proteins expressed. The supportive cells are the most specialized, for example, glial cells in the CNS and stroma cells in the endometrium and ovary. In contrast, the study showed that several glandular cells have as many as 80% of the analyzed proteins present at detectable levels, and this raises the question how much the result is influenced by background staining due to nonspecific binding or cross-reactivity to homologous proteins. To explore this issue, a sensitivity analysis was carried out, using various subfractions of the antibodies (see Supplementary Table S1) with the selection based on paired antibodies with high

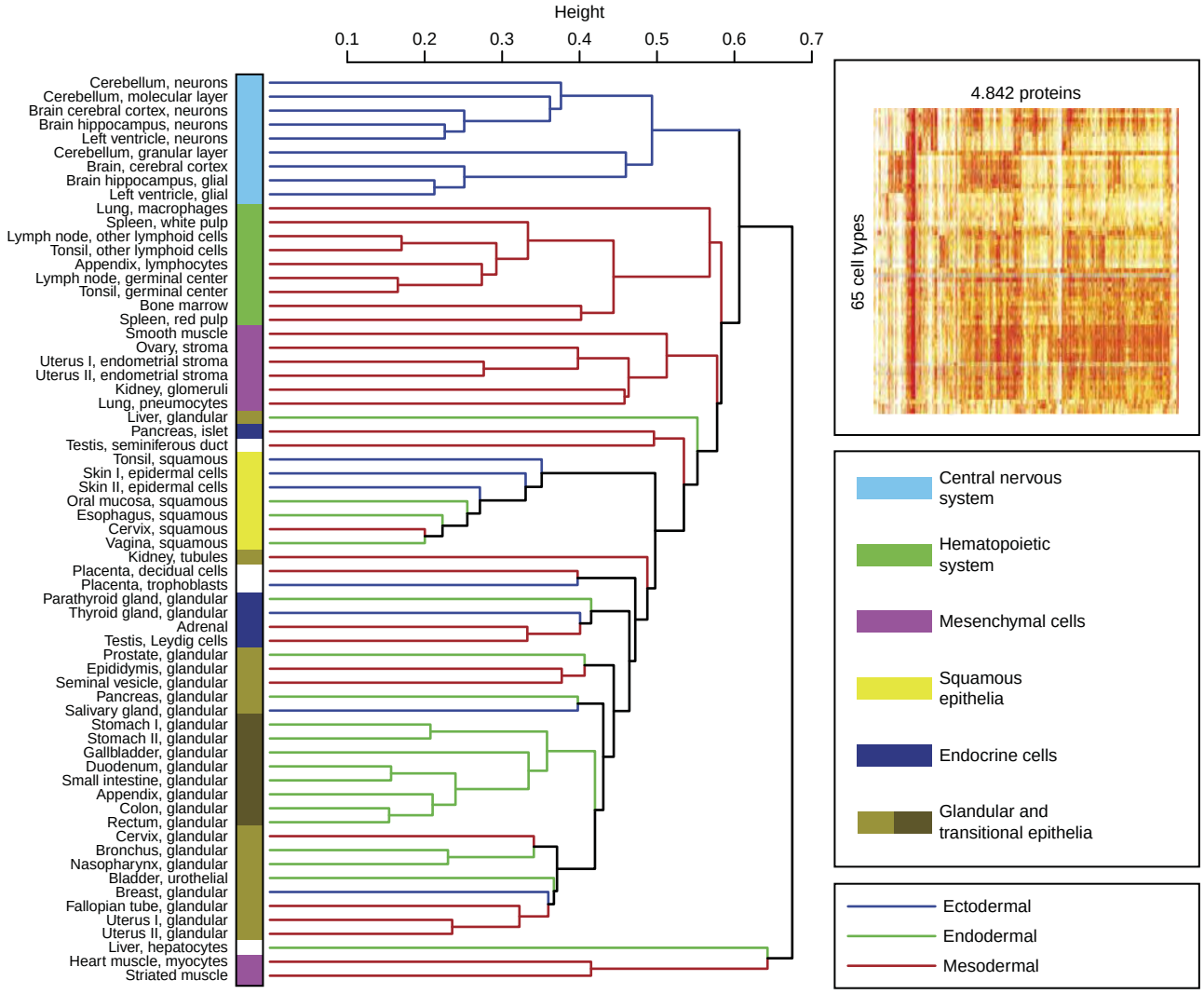

Figure 1 Global protein profiling in 65 normal human cell types. A dendrogram showing the relationships, based on global expression profiling, between the various cell types. The dendrogram was constructed using a hierarchical clustering model; the inset shows the original heat map. The underlying data are based on manual annotation of protein expression patterns in 65 normal cell types using 5934 antibodies corresponding to 4842 proteins. The dendrogram bars are labeled according to the proposed origin within the embryonic germ cell layers: ectoderm (blue), mesoderm (red), and endoderm (green). The cell types have been classified into six categories according to the color code to the right. A list of all the cell types can be found in Supplementary Table S2. The dendrograms for proteins encoded on single chromosomes are shown in Supplementary Figures S1–S3 and for random sets of 200 antibodies in Supplementary Figures S4–S6.

confidence or documented supportive western blots. The analysis showed essentially the same results as when all antibodies were used (Supplementary Figures S7, S8 and Table S2), with an average of 61–71% of the proteins detected across the 65 cell types.

Differential expression in selected human cell types

A study was performed to explore the difference in protein expression in three cells with distinctly different phenotypes, namely hepatocytes from the liver, neurons from the cerebral cortex of the brain, and lymphoid cells from the germinal center of the lymph nodes. A network analysis (Shannon *et al*, 2003) showed (Figure 2C) that as much as 90% of the antibodies (n=5138) detect proteins that are expressed in at least one of the three cells and few proteins are detected exclusively in one of the cells, as exemplified by the brain (9%). However, the cells still display a highly differentiated global expression pattern as shown by the fact that only 6% of the proteins are expressed at the same level in all three cell types. We extended this study to explore the protein profiles in three more closely related cell types: glandular cells in the colon, epidermal cells from the skin, and urothelial cells from the bladder. In this case, a smaller fraction (59%) of the antibodies (n=3376) detect proteins in all three cells and a larger fraction (17%) of the proteins were scored with the same expression level in all three cells (Figure 2D). However, considering the fact that these cells share a common epithelial phenotype, it is interesting that a large fraction (74%) of the proteins still have differential

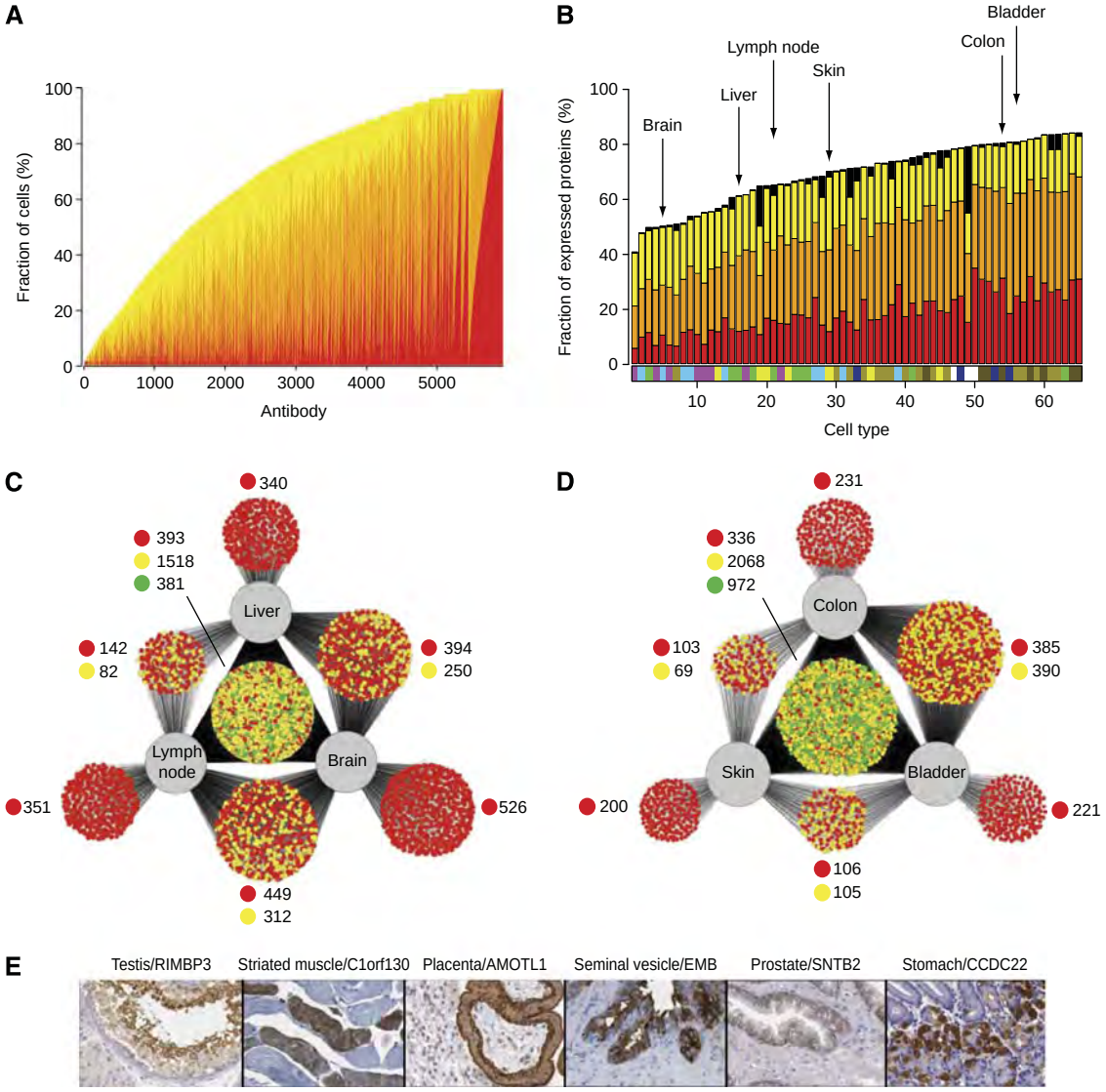

Figure 2 The tissue-specific protein expression in 65 cell types corresponding to 48 tissues and organs. (**A**) The fraction (%) of cells in which a particular protein was detected, including the fraction of cells with the relative expression levels strong (red), moderate (orange), and weak (yellow). A total of 5934 antibodies against 4842 proteins are arranged according to abundance of the corresponding protein target with cell-type-specific proteins to the left and 'housekeeping proteins' to the right. The results for the various subfractions of antibodies are presented in Supplementary Figure S7. (**B**) The fraction (%) of the analyzed proteins detected in a specific cell type. Cells are arranged according to the fraction of proteins detected. A bar displaying the different color codes representing the six major categories of normal cell types (defined in Figure 1) is shown for each cell type. The name of each cell type is shown in Supplementary Table S2 and the results for the various subfractions of antibodies are presented in Supplementary Figure S8. Black represents missing data, i.e., where there was no representative cell type for a given immunostaining. The six cell types analyzed in C and D are pointed out by arrows. (**C**) A Cytoscape network plot (Shannon *et al*, 2003) showing the distribution of the analyzed proteins detected in at least one of the three cell types analyzed; liver hepatocytes, neurons of the cerebral cortex of the brain, and lymphoid cells from the germinal center of the lymph node. Each antibody/protein is represented by a small circle that is connected by a line/lines to the cells it was detected in. The color of the circle indicates the variability of staining intensity between the different cell lines; green indicates that all cell lines belonged to the same staining intensity category, yellow indicates that two cell lines belonged to the same staining intensity category, and red indicates that the staining intensity category was different for all three cell lines or only detected in a single cell. (**D**) A similar network plot based on the analysis of protein expression in glandular cells in the colon, epidermal cells from the skin, and urothelial cells from the bladder. (**E**) Six examples of proteins with essentially unknown functions that exhibit cell-type-specific expression. Testis—maturing spermatocytes and spermatids in the testicular seminiferous duct show strong partly membranous positivity with an antibody generated toward the uncharacterized protein RIMS-binding protein 3A. Muscle—striated skeletal muscle is shown with a fiber-type-specific sarcoplasmic positivity with an antibody directed toward an unknown protein encoded by C1orf130. Placenta—the expression of angiomotin-like protein 1 in placental tissue (immature) shows strong membranous positivity in basal cytotrophoblasts with moderate cytoplasmic positivity in syncytiotrophoblasts and exhibits distinct expression in the brush-border membrane. Seminal vesicle—glandular cells in the seminal vesicle were the only cells found to express embigin, a previously unknown protein. Prostate—in the prostate, moderate positivity was found with a membranous expression pattern for beta-2-syntrophin protein (SNTB2), a protein with unknown functions that has been shown to co-purify, with dystrophin, the protein product of the Duchenne muscular dystrophy locus. Stomach—in the stomach mucosa the previously unknown coiled-coil domain-containing protein 22 (CCDC22) was expressed in the parietal cells, producers of hydrochloric acid in response to histamine, acetylcholine, and gastrin.

expression across the three cell lines. As expected, cells with more similar functions show less differences in global protein expression patterns compared with those with widely different functions. Despite the high fraction of overall expression of proteins, the given examples show that only 6% of expressed proteins are expressed at the same level in widely different cell types compared with 17% in more closely related cell types. The network analysis provides a further insight into the dynamics of cellular phenotypes and functions as a consequence of differences in protein signatures and allows for a novel angle to determine which cell types are most similar (and most dissimilar) irrespective morphological traits and functions.

Analysis of tissue-specific proteins

To identify tissue-specific proteins, we analyzed how many proteins could be detected exclusively in a single cell and this query resulted in a list of 74 proteins (Supplementary Table S4). The list included several previously well-known cell-type-specific proteins, such as insulin, glucagon, IAPP (Langerhans islets), troponins (muscle), PSA, ACPP (prostate), and several CD markers (hematopoietic cells). The analysis also identified a subset of cell-type-specific proteins for which there is no or little information, including proteins exclusively expressed in the testis, skeletal muscle, placenta, seminal vesicle, prostate, and stomach (Figure 2E). Several of these proteins showed a remarkable specificity, with expression in only a subset of the entire annotated cell population, for example, expression in parietal cells of the stomach mucosa and fiber-type-specific expression myocytes. Expanding the query to include similar cell types at different locations, for example, the nine annotated cell populations in the brain, also showed a surprisingly low number (n=30) of proteins exclusively expressed in the brain (data not shown). These results are somewhat surprising considering the numerous reports describing genes expressed in a tissue-specific manner (Saito-Hisaminato *et al*, 2002), in particular examples of genes exclusively expressed in the brain, such as the KIAA genes (Ishikawa *et al*, 1997) In summary, our analysis shows a surprisingly low fraction (<2%) of proteins expressed in a single or only few distinct types of cells. The few cell- or tissue-specific proteins that were found are, of course, interesting starting points for further studies and this is facilitated by the fact that all the annotation results and the underlying original images are available as a public resource from the Human Protein Atlas portal.

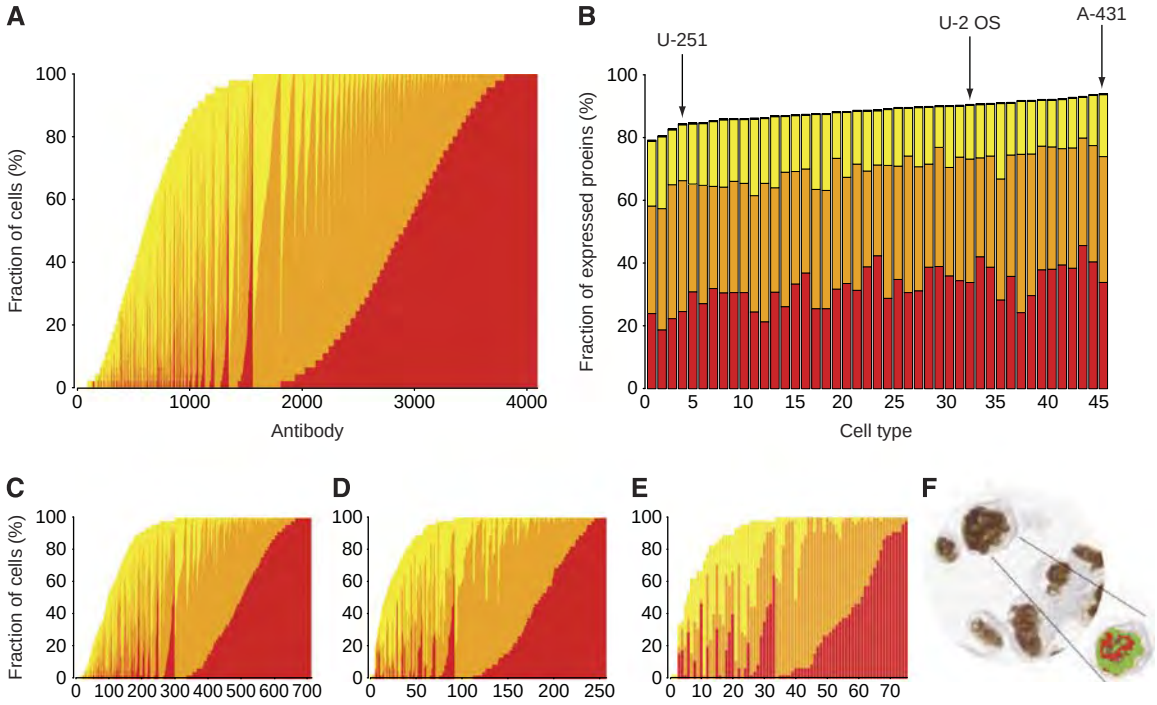

Figure 3 Global protein expression in 45 human cell lines. (**A**) The fraction (%) of the 45 cell lines in which a particular protein was detected, including the fraction of the three relative expression levels: strong (red), moderate (orange), and weak (yellow). Each bar represents one of the 4096 antibodies with no missing data, i.e., where all cell lines were represented. (**B**) The fraction (%) of a total number of 5349 antibodies against 4349 proteins detected in a specific cell line, and with the cell lines ordered according to the fraction of proteins detected. The corresponding name and number of each cell line is shown in Supplementary Table S3 and the results for the various subfractions of antibodies are presented in Supplementary Figure S9. The same three staining categories were used and the black (top) part of the bar represents antibodies with missing data for the particular cell line. Arrows point out the three cell lines used in immunofluorescence analysis. (**C**) The fraction of cell lines in which each protein from a data set of 714 antibodies with supportive results from western blot analysis was detected. (**D**) A plot similar to C, with each bar representing one of the 257 antibodies remaining from a data set of paired HPA-antibodies, i.e., toward the same target protein, with no missing data for any of the cell lines, and a correlation coefficient of $\geqslant$0.5 when cell line expression profiles were analyzed. (**E**) Same as D, but displaying only the results from the 75 antibodies with a correlation coefficient of $\geqslant$0.8 and no missing data. (**F**) An example of cells, visualizing the interpretation of immunostaining by an automated image analysis software.

Global protein expression in 45 human cell lines

As all the immunohistochemical images from the TMAs were manually annotated by pathologists involving subjective scoring, we decided to carry out the same analysis on 45 human cell lines in which an automated image analysis algorithm have been used (Stromberg *et al*, 2007; Lundberg *et al*, 2008). The data from 5349 antibodies corresponding to 4349 genes were analyzed, involving more than 450 000 additional images, and the results are shown in Figure 3. A pattern of protein expression similar to that for tissues and organs was recorded for the *in vitro* cultured cells, with most proteins expressed in the majority of the 45 cell lines (Figure 3A) and nearly 80% of the proteins expressed across all the analyzed human cell lines (Figure 3B and Supplementary Table S3). A sensitivity analysis using antibodies with supportive western blots (Figure 3C) or paired antibodies with highly correlated expression patterns (Figure 3D and E, and Supplementary Figure S9) produced similar results. An example of the automated image analysis algorithm can be seen in Figure 3F.

Global protein expression in cell lines using confocal microscopy

The immunohistochemical analysis, based on an enzyme amplification method, is semiquantitative and we therefore decided to extend the study using immunofluorescence analysis with confocal microscopy. An analysis of three selected human cell lines (Barbe *et al*, 2008) of epithelial (A-431), glial (U251-MG), and mesenchymal (U-2 OS) origin was carried out for 2,064 proteins (see Figure 4C). More than 70% of the proteins were detected in each of the three cell lines (Figure 4A), even when proteins without a defined subcellular localization (i.e., with weak, granular, and cytoplasmic staining) were excluded. Only 14% of the proteins could not be detected in any of the three cell lines. A plot was generated

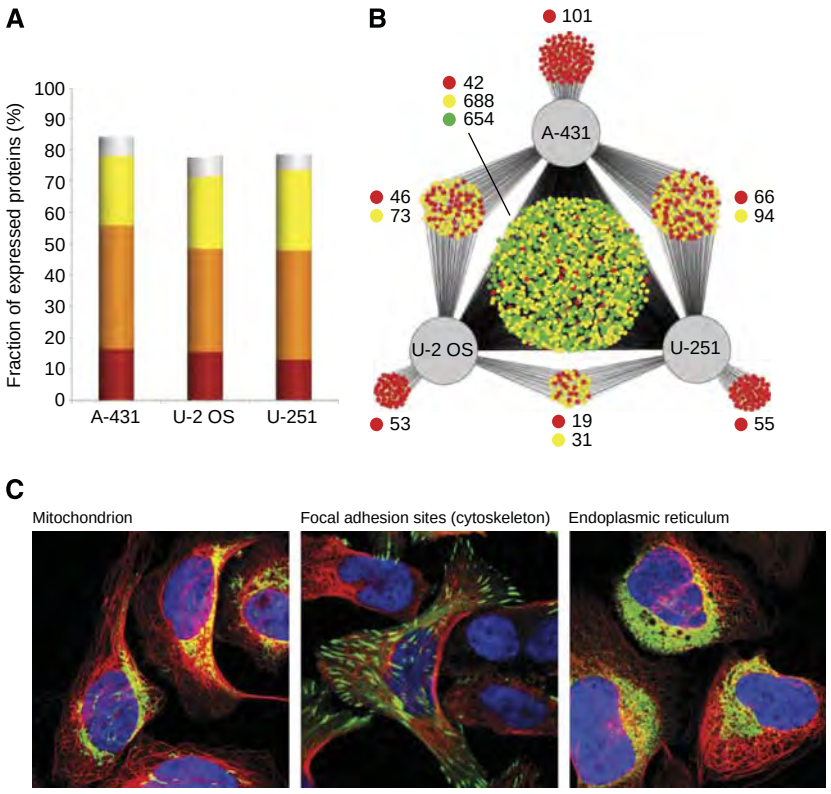

Figure 4 Global protein expression in three human cell lines using immunofluorescence-based confocal microscopy. (**A**) The fraction (%) of proteins detected in the three analyzed cell lines. The stainings are classified into the categories strong (red), moderate (orange), and weak (yellow) based on their measured intensity. Stainings annotated as weak with a granular cytoplasmic subcellular distribution (i.e., not a distinct cytoplasmic organelle or component) are considered less reliable (shown in gray). (**B**) A Cytoscape network plot (Shannon *et al*, 2003) showing the distribution of the analyzed proteins detected in at least one of the three cell lines. Each antibody/protein is represented by a small and the color of the circle indicates the variability of staining intensity between the different cell lines; green indicates that all cell lines belonged to the same staining intensity category, yellow indicates that two cell lines belonged to the same staining intensity category, and red indicates that the staining intensity category was different for all three cell lines. (**C**) Three example images of immunofluorescently stained U-2 OS showing proteins from the different categories (green, yellow, and red) in panel B and with different subcellular localizations. The protein of interest is shown in green, microtubules in red, and nuclei in blue. The first image (left) shows the 60-kDa heat shock protein (HSPD1) to be localized in the mitochondria and give a strong staining intensity in all three cell lines (green category) as detected by the antibody HPA001523. The second image (middle) shows the four and a half LIM domains protein 2 (FHL2) to be localized at focal adhesion sites in the cytoskeleton and give a strong staining intensity in U-2 OS and a moderate staining intensity in A-431 and U-251 MG (yellow category) as detected by the antibody HPA006028. The third image (right) shows the uncharacterized protein KIAA1467 to be localized in the endoplasmic reticulum and give a strong staining intensity in U-2 OS, moderate in A-431, and weak in U-251 MG (red category) as detected by the antibody HPA010803.

to show the number of proteins expressed in one, two, or all cell lines (Figure 4B), and the results show that the majority (72%) of the antibodies (n=1384) detect proteins in all three cell lines and that only 11% of the proteins are detected in just one out of the three cell lines. As to the tissue analysis (Figure 2C and D), the majority (66%) of the proteins were expressed at a different level in at least one of the three cell lines.

Conclusions

Our findings suggest that few proteins are expressed in a cell-type-specific manner, and that the phenotype and function of a cell is determined by localization and fluctuations in concentration of a large portion of the proteome, as opposed to a binary 'on/off' expression pattern. Although antibody-based assays are sensitive, it is possible that even more sensitive assays, such as the use of sandwich-based analysis (Larsson *et al*, 2004), could allow the detection of lower levels of proteins, thus showing an even more ubiquitous expression. It is important to point out that the regulation of proteins is also mediated through protein modification, in which certain fractions of the proteins are activated by chemical modification (Olsen *et al*, 2006) or proteolysis (Yen *et al*, 2008). The separate functions of a particular cell are, thus, a consequence of local concentrations and modifications of proteins that are carefully regulated to ensure proper functionality in each cell type. For example, the concentrations of a majority of our proteins in a human kidney cell provide an interaction network appropriate for kidney functions (filtration), whereas the protein interaction network in a specific neuron in the brain is targeted toward neurological functions. These data therefore suggest that the phenotype of a particular cell is a consequence of the local concentration of a large portion of the human proteome. This underlines the importance of systems biology approaches (Kislinger *et al*, 2006) based on quantitative measurements of protein levels (Cox and Mann, 2008) and network predictions of protein interactions (Shlomi *et al*, 2008) to study mammalian biology. In this context, it would be interesting to add quantitative expression data from complementary technology platforms, such as mass spectrometry (Mann and Kelleher, 2008), to further explore the protein space in individual cells. The analysis could also be integrated with numerous RNA-based expression studies (Kilpinen *et al*, 2008) to gain further in-depth understanding of the relationship between transcript and protein profiles. To facilitate such bioinformatics comparisons, the expression data used in this analysis are available for downloads at the public Human Protein Atlas portal (http://www.proteinatlas.org/download.php). In conclusion, this study suggests that tissue specificity is achieved by precise regulation of protein levels in space and time, and the results emphasis the need for quantitative systems biology approaches to understand the molecular mechanisms of human biology and diseases.

Materials and methods

Data collection and extraction

To determine the level of protein expression of each protein in this study, antibodies were used to immunohistochemically stain human tissues assembled in TMA blocks (Kononen *et al*, 1998). Tissue cores with 1 mm diameter, sampled from 144 individuals, corresponding to 48 different normal human tissues types, were included in the study. In addition, a microarray containing human cell lines was assembled (CMA) (Kampf *et al*, 2004). In addition, the protein levels were estimated for three human cell lines (A-431, U-2OS, and U-251 MG) using immunofluorescence-based confocal microscopy (Barbe *et al*, 2008). Immunohistochemically stained sections from TMA/CMA blocks were scanned in high-resolution scanners and separated to individual spot images representing each core. For TMAs, all images were evaluated by certified pathologists in a web-based annotation system to collect parameters regarding distribution, the extent and level of protein expression (P Oksvold and E Björling, unpublished results). Parameters from the annotation included staining intensity, fraction of stained cells in a defined cell population, and subcellular localization of staining. The annotation was performed for selected cell types for each tissue, as most tissue types include several defined cell phenotypes, e.g., neurons and glial cells in brain tissue and glomeruli and tubules in the kidney (Bjorling *et al*, 2008). CMAs were evaluated using automated image analysis (Stromberg *et al*, 2007) where five output parameters were combined to calculate a score for the protein expression level. For immunofluorescence images, subcellular localizations were annotated and the relative expression levels were classified as strong, moderate, weak, or negative based on the employed laser power and detector gain settings.

In total, 5934 antibodies against 4842 proteins with 298 annotations were assembled from TMA measurements and 5349 antibodies against 4349 proteins with 45 annotations from CMAs. The annotation parameters for intensity and quantity (fraction of positively stained cells) were combined into a four-grade scale represented by the colors white (negative), yellow (weak), orange (moderate), and red (strong) level of protein expression. All data are presented in this format on the protein atlas (http://www.proteinatlas.org). For statistical analysis regarding protein expression, the color codes representing the staining levels were converted to numerical values using a red to 4, orange to 3, yellow to 2 and white to 1 transformation. In cases where the protein expression value could not be derived, because of low image quality, a not available (NA) value was introduced. These data were ordered into a matrix with m (number of antibodies) $\times$ n (number of tissues (n=65) or cell lines (n=45)) dimensions. The number of tissues is a combined tissue and cell type parameter, where the number of tissues and cell types give rise to $\times$ the number of tissues cell type parameters. In all, two matrices were constructed; one matrix contains protein expression data from human normal tissues and a second matrix contains protein expression data from human cell lines. The number of antibodies in the two matrices and all subsets used in the different figures is presented in Supplementary Table 1.

Hierarchical clustering

For the normal tissue data set, two correlation matrices based on Spearman's ρ were calculated for two dimensions ($m \times m$ and $n \times n$, respectively) (Spearman, 1987). The correlation matrices were converted to a distance metric using a 1−correlation value transformation. These data were clustered using unsupervised top-down hierarchical clustering (Eisen *et al*, 1998; Golub *et al*, 1999), where at each stage the distances between clusters are recomputed by the Lance–Williams dissimilarity update formula according to average linkage. The algorithm consistently sorted the tighter cluster in each division to the left in the resulting dendrogram representing the hierarchical cluster output. The antibodies with no defined correlation due to constant expression across all tissues or cell lines were removed in the clustering procedure.

Statistical analysis

To estimate protein expression values for each protein across all tissues and cell lines, the different intensity categories (weak, moderate, and strong) were added as separate units into a marginal distribution, which constitutes of 4 $\times$ the number antibodies values. The marginal distribution can be seen as a proxy for the total expression level for the respective protein across the 65 tissues and cell types used in this study. A similar procedure was conducted for the protein expression

for each tissue and cell line across all antibodies, resulting in a marginal distribution of 4 × the number of tissues or cell lines values.

Validity estimation of dendrograms and marginal distributions

In order to investigate the quality and the conclusions made from data, different subsets were constructed and used in the same analysis approaches as the full data set. For the hierarchical clustering, three additional data sets were constructed, where the subsets were selected based on chromosomal appurtenance using 215, 203, and 206 antibodies representing proteins from chromosome 9, 10, and 22 respectively. The similarity between the dendrograms generated with the three subsets and the dendrogram using all antibodies were investigated using cophenetic correlation coefficients (Sokal and Rohlf, 1962). To estimate the reliability of the marginal distributions, different subsets of antibodies were chosen (Supplementary Table S1). One of the subsets was chosen based on western blot data, where the expected size of the protein matches the correct band from a western blot gel image. Two additional subsets were built based on correlation analysis of paired antibodies, where the two antibodies in a pair are generated to different parts of the same protein. For each antibody pair, a correlation coefficient was calculated using Spearman's ρ. A cutoff value of 0.5 was applied to construct subsets used for the tissue and cell type data set and for the human cell lines. A cutoff of 0.8 was implemented to construct an additional data set for the human cell lines. To estimate the similarities of the sub sets and the full data set, a χ^2 test statistic was used on the marginal distributions.

Network analysis

In order to visualize the protein expression overlap between different tissues or cell lines, Cytoscape (Shannon *et al*, 2003), a software package for analyzing biomolecular interaction networks, was used. The resulting images contain schemes that indicate the overlap of different proteins across different sets of cell types. The first combination was hepatocytes from the liver, neurons from the cerebral cortex of the brain, and lymphoid cells from the germinal center of the lymph nodes, where all three cell types have distinctly different phenotypes. The second combination consisted of three more closely related cell types, namely glandular cells in the colon, epidermal cells from the skin, and urothelial cells from the bladder. The third group of cells consisted of the three cell lines used for immunofluorescence analysis, namely A-431, U-2OS, and U-251 MG. Antibodies with missing protein expression data for any of these cell types were removed from the analysis. This resulted in three different sets of 5710, 5700, and 2250 antibodies, respectively. In each of the resulting networks, a node represents an antibody with a specific protein expression profile, and the edges connect the node to the cell(s) where certain protein is expressed, generating nodes with a degree (number of edges) of 1, 2, or 3. Thus, only antibodies corresponding to proteins expressed in the analyzed cell types are parts of the network. The total number of nodes in the three networks was 5138, 5186, and 1921. Each node was colored according to the variability of staining intensity between the analyzed cell types. Red nodes indicate different staining categories for the three cell types, yellow nodes indicate two cell types in the same staining category, and green nodes indicate that all three cell types belong to the same staining category and are therefore only found for nodes with a degree of three.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 19

Confocal Microscopy of Live Cells

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By

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CHAPTER 19

CONFOCAL MICROSCOPY OF LIVE CELLS

M.E. Dailey, E. Manders, D. Soll, and M. Terasaki

INTRODUCTION

If a picture is worth a thousand words, then a movie may be worth a million words. Microcinematography and, later, video microscopy have provided great insight into biological phenomena. One limitation, however, has been the difficulty of imaging in three dimensions. In many cases, observations have been made on cultured cells that are thin to start with or tissue preparations that have been sectioned.

The development of the first beam-scanning confocal microscope was motivated by the goal of making observations in the tissues of living organisms (Petran et al, 1986). The optical sectioning capability of the confocal or multiphoton (MP) microscope allows one to make thin-slice views in intact cells or even intact animals. Confocal microscopes are now fairly common, and because they employ non-ionizing radiation, they are increasingly being used to study living cells and tissue preparations.

What are the specific challenges of applying confocal imaging to studies of living cells? First, the experimenter must do no harm. Arguably, the main obstacle in living cells microscopy is not "getting an image" but doing so without upsetting the cell. To be useful, the study must be carried out on a biological system that retains normal function and can be subjected to controlled conditions while on the stage of the microscope. Often, environmental variables such as temperature, CO_2, or pH must be regulated and/or an efficient directional perfusion system must be used. Unfortunately, the difficulty of keeping cells alive and functioning on the microscope discourages many researchers. This chapter is designed to help them succeed well enough to become convinced of its importance and utility.

Other difficulties are more specific to confocal fluorescence microscopy. All studies with fluorescence benefit from collecting as much of the emitted fluorescent light as is possible, but this is particularly important for studies of living cells because photodynamic damage and consequent alteration in normal cell behavior is a very real possibility (see Chapters 16 and 38). Therefore, optimizing microscope photon collection efficiency is crucial for successful confocal microscopy of living cells.

Another difference between living and fixed cell studies is the element of time. All living processes have an inherent time course, and the imaging system must produce images at the appropriate rate to show the changes involved. The amount of light necessary to obtain the data must be apportioned over time so that enough images can be obtained to describe the process under investigation without damaging the cells. Although early confocal microscopes had a relatively slow scan speed, newer technology now permits very rapid image collection to explore spatially and temporally dynamic biological processes (see Chapter 10). In single-beam scanning systems, the field of view often is reduced to achieve higher imaging speeds.

Lastly, the fluorescent probes used in studies of living cells must not impair normal cell function. Immunofluorescence, which has been used so successfully to localize molecules in fixed cells, has not been

practical in living cells. However, there are now many commercially available fluorescent probes for structural and physiological studies of cells and tissues (see Chapters 16 and 17). Of even more importance, the 'green revolution' based on the green fluorescent protein (GFP) has changed the landscape and is ushering in an exciting period of biological imaging of proteins in living cells, and of various cell types in living, intact tissue preparations (Chalfie et al, 1994; Bastieans & Pepperkok, 2000).

Although confocal microscopy of living cells is difficult, its usefulness was demonstrated over 15 years ago in two pioneering studies. Cornell-Bell et al. (1990) used confocal microscopy to make a major discovery: the existence of glutamate-stimulated, transcellular Ca^{2+} waves in astroglia. In the same year, confocal microscopy was used to characterize developmental changes in an intact animal by imaging neuronal axons and their growth cones in the developing brain of a tadpole (O'Rourke and Fraser, 1990). Ever since these pioneering studies, there has been an increasing use of confocal microscopy to study dynamic processes in an array of diverse biological preparations. When the second edition of this volume appeared in 1995, about 80 papers using confocal microscopy on living cells were found. Today (early 2005), there are over 500 published studies using live -cell confocal imaging.

While live -cell applications of confocal imaging have expanded significantly over the past decade, multiphoton (MP) imaging (see Chapters 28 and 37) is poised to make a similar impact on live -cell and tissue studies in the next decade. There are trade-offs, however. MP imaging can be useful especially for very deep penetration in tissues (>100μm) where non-descanned detection increases signal substantially, but from thinner specimen, the actual damage/excitation may be greater for MP than for single photon confocal imaging (Tauer, 2002; see also Chapter 38). Moreover, the cost differential is such that one could have 2–3 graduate students working away on disk scanners or simpler beam scanning confocal units for every one on a MP unit. At any rate, most of the topics covered in this chapter are relevant for both single photon confocal and MP excitation.

OVERVIEW OF LIVE -CELL CONFOCAL IMAGING TECHNIQUES

Although live -cell imaging often involves time-lapse microscopy to monitor cell movements, modern approaches are extending these observations well beyond simply making movies of cell structure. Increasingly, time-lapse imaging is being integrated with specialized techniques for monitoring, measuring, and perturbing dynamic activities of cells and subcellular structures. Below we summarize some major techniques available for studying the dynamic organization of molecules and cells in live biological specimen. These techniques are summarized in **Table 19.1.**

a. Time-lapse fluorescence imaging.

Time-lapse fluorescence imaging involves repeated imaging of a labeled specimen at defined time points, thereby permitting studies on the dynamic distribution of fluorescently labeled components in living systems. Imaging can be performed in one, two, or three spatial dimensions: 1-D imaging involves rapid

and repeated imaging of single scan lines; 2-D imaging involves repeated imaging of single focal planes; and 3-D imaging involves repeated imaging of multiple focal planes in thick specimen. The time intervals for sequential image collection can range from sub-second to days or even months (e.g., Gan et al., 2003).

Many small-molecule, vital fluorescent probes that give highly specific cellular or subcellular patterns of labeling are now available (see below and Chapters 16 and 17). In addition, GFP or GFP-related proteins are now routinely fused to other proteins of interest, and the inherent brightness and photostability of many of these fluorescent proteins make them well suited for the repeated imaging needed for time-lapse studies. Together, these fluorescent probes are affording a seemingly limitless array of possibilities for imaging molecular components in live cells.

b. Multi-channel time-lapse fluorescence imaging.

The plethora of excellent vital fluorescent labels with varying spectral characteristics (including spectral variants of GFP) allows multi-label experiments to visualize the relative distribution of several different cell or tissue components simultaneously. Advances in imaging technology have facilitated automated collection of more than one fluorescent channel (either sequentially or simultaneously) with improved ability to maximize signal collection and to separate partially overlapping signals.

In addition to studies using multiple fluorescent tags, multi-channel data collection permits ratiometric imaging of single probes whose spectral properties (absorption or emission) change depending on ionic conditions, such as the Ca^{2+} sensitive physiological indicator, indo-1 (see Chapter 42).

c. Spectral imaging and linear unmixing.

Increasingly, experiments are incorporating multiple fluorescent probes within single cells or tissues to define the differential distribution of more than one labeled structure or molecular species. Such multi-color or multi-spectral imaging experiments require adequate separation of the fluorescent emissions, and this is especially problematic when the spectra are substantially overlapping. Spectral imaging utilizes hardware to separate the emitted light into itsspectral components. Linear unmixing is a computational process related to deconvolution that uses the spectra of each dye as though it were a point-spread function of fixed location to "unmix" the component signals (Tsurui et al., 2000; Lansford et al., 2001; Hiraoka et al., 2002). Although together, these analytical tools can be used to discriminate distinct fluorophores with highly overlapping spectra (Zimmermann et al., 2003), they do so at the cost of requiring that significantly more photons be detected from each pixel.

d. Fluorescence recovery after photobleaching (FRAP).

Fluorescence recovery after photobleaching (FRAP), also known as fluorescence photobleaching recovery (FPR), is a technique for defining the diffusion properties of a population of fluorescently labeled molecules (Axelrod et al., 1976; Koppel et al., 1976; for review, see Lippincott-Schwarz et al., 2003).

Typically, a spot or line of intense illumination is used to bleach a portion of a fluorescent cell, and the recovery of fluorescent signal back into the bleached area from adjacent areas is monitored over time (usually seconds to minutes). Although this technique can yield quantitative information on the diffusion coefficient, mobile fraction, and binding/dissociation of a protein, care has to be taken not to use so much power in the bleach beam that the cellular structure is disrupted (Bloom & Webb, 1984; Flock et al., 1998). Quantitative assessments of FRAP data, which can be confounded by uncertainties in the experimental and biological parameters in living cells, may benefit from computer simulations (Weiss, 2004).

e. Fluorescence loss in photobleaching (FLIP).

This technique utilizes repeated photobleaching in an attempt to bleach all fluorophore within a given cellular compartment (Lippincott-Schwarz et al., 2001). Thus, FLIP can be used to assess the continuity of membrane bounded compartments (e.g., ER or Golgi Apparatus) and to define the diffusional properties of components within, or on the surface of, these compartments.

f. Fluorescence resonance energy transfer (FRET).

Fluorescence resonance energy transfer (FRET) is a technique for defining interactions between two molecular species tagged with different fluorophores (Stryer, 1978; Sekar & Periasamy, 2003). It takes advantage of the fact that the emission energy of a fluorescent "donor" can be absorbed by (i.e., transferred to) an "acceptor" fluorophore when these fluorophores are in nanometer proximity and have overlapping spectra (see Chapters 16, 27 and 45).

g. Fluorescence lifetime imaging (FLIM).

This technique measures the lifetime of the excited state of a fluorophore (Lakowicz et al., 1992 and Chapter 27). Each fluorescent dye has a characteristic "lifetime" in the excited state (usually 1-20 nanoseconds), and detection of this lifetime can be used to distinguish different dyes in samples labeled with multiple dyes. FLIM can be utilized in conjunction with FRET analysis because the lifetime of the donor fluorophore is shortened by FRET. In fact, FLIM can improve the measurement during FRET analysis because the fluorescence lifetime is independent of the fluorophore concentration and excitation energy (Bastiaens & Squire, 1999; Elangovan et al., 2002; Chen et al., 2003, and Chapter 27). However, the lifetime can be modulated by environmental considerations (e.g., pH, ion concentration), and this change can be used to measure changes in the concentration of certain ions (Lin et al., 2003).

h. Fluorescence correlation spectroscopy (FCS).

Fluorescence correlation spectroscopy (FCS) measures spontaneous fluorescence intensity fluctuations in a stationary microscopic detection volume (about 1 femptoliter) (Magde et al., 1974). Such intensity fluctuations represent changes in the number or quantum yield of fluorescent molecules in the detection

volume. By analyzing these fluctuations statistically, FCS can provide information on equilibrium concentrations, reaction kinetics, and diffusion rates of fluorescently tagged molecules (Elson, 2001). An advantage of this approach is the ability to measure the mobility of molecules down to the single molecule level and to do so using a light dose orders of magnitude lower than used for FRAP.

i. Fluorescence speckle microscopy (FSM).

The dynamic growth and movement of fluorescently labeled structures can be difficult to analyze when these structures are densely packed and overlapping within living cells. Fluorescent speckle microscopy (FSM) is a technique compatible with widefield or confocal microscopy (Adams et al., 2003) that uses a very low concentration of fluorescently labeled subunits to reduce out-of-focus fluorescence and improve visibility of labeled structures and their dynamics in thick regions of living cells (Waterman-Storer et al., 1998). This is accomplished by labeling only a fraction of the entire structure of interest. In that sense, it is akin to performing FCS over an entire field of view, albeit with more focus on spatial patterns than on quantitative temporal analysis. FSM has been especially useful for defining the movement and polymerization/depolymerization of polymeric cytoskeletal elements, such as actin and microtubules, in motile cells (Salmon et al., 2002).

j. Photo-uncaging/Photo-activation.

Photo-uncaging is a light-induced process of releasing a 'caged' molecule from a caging group to produce an active molecule (Politz, 1999; Dorman & Prestwich, 2000). A variety of caged molecules have been synthesized and used experimentally, but in some instances cages have been used to mask a fluorophore, inducing a non-fluorescent state. Excitation light of ~350nm is used to break photolabile bonds between the caging group and fluorophore, thereby uncaging the fluorophore and yielding a fluorescent molecule.

A related technique utilizes genetically encoded, photo-activatable fluorescent proteins, of which there are currently about a dozen (for review, see Patterson & Lippincott-Schwartz, 2004). Two examples include a photo-activatable (PA) form of GFP, called PA-GFP (ex/em: 504/517), which shows a 100-fold increase in fluorescence following irradiation at 413 nm (Patterson & Lippincott-Schwartz, 2002), and Kaede (ex/em: 572/582), which shows a 2,000-fold increase following irradiation at 405 nm (Ando et al., 2002).

An extension of the photo-activation approach, termed reversible protein highlighting, has been developed (Ando et al., 2004). This involves reversible, light-induced conversion of a coral protein, Dronpa, between fluorescent and non-fluorescent states. One study used this approach to monitor fast protein dynamics in and out of cell nuclei (Ando et al, 2004). Thus, photo-uncaging and photo-activation are complementary to FRAP and can be used in conjunction with time-lapse imaging to mark and follow a population of molecules in order to study their kinetic properties within living cells.

k. Optical tweezers/laser trapping.

Optical tweezers, or single beam laser trap, uses the 'radiation pressure' of a stream of photons emitted from an infrared laser to "trap" small objects (often a protein-coated bead) and to move them around (Sheetz, 1998; Kuo, 2001 and Chapters 5 and 9). This technique has been especially useful for quantifying forces generated by motor protein movement (Ashkin et al., 1990; Block et al., 1990; Kuo & Sheetz, 1993) or the strength of adhesions mediated by cell adhesion molecules (e.g., Schmidt et al., 1993; Baumgartner et al., 2003). Although "laser tweezers" often are used in widefield imaging systems, they also have been incorporated into confocal (Visscher & Brakenhoff, 1991) and MP (Goksor et al., 2004) imaging systems.

l. Physiological fluorescence imaging.

The availability of fluorescent physiological indicators extends live -cell confocal and MP imaging studies beyond structural aspects to study cell and tissue physiology (Niggli & Egger, 2004; Rubart, 2004; Wang et al., 2004). Calcium indicators have been the most commonly used physiological probes because calcium is a central signal transduction molecule and in many cell preparations the calcium-sensitive probes give robust signals. These signals often are temporally resolvable in full field scans as calcium transients that persist for several seconds. Fast scanning systems, or line-scanning mode in laser scanning systems, have been used to resolve more rapid calcium events (e.g., Fan et al., 1999; Wang et al., 2004). Although non-ratiometric, visible wavelength calcium indicators (e.g., fluo-3, calcium green) have been more widely used in confocal applications, some studies have employed UV excited ratiometric calcium indicators, such as indo-1 (e.g., Pasti et al., 2001).

In addition to calcium indicators, other fluorescent physiological probes are useful for reporting various ions including sodium, magnesium, potassium, and chloride, pH, heavy metals such as zinc, and membrane potential, to name a few (see Chapter 42). Although many of these probes are small molecules, genetic (GFP-based) probes have been developed (see Miyawaki, 2003) and are being incorporated into transgenic animals (e.g., Hasan et al., 2004). In combination with state-of-the-art confocal and MP imaging systems, these probes will increasingly permit detailed spatio-temporal analyses of physiological processes in intact tissues and organisms (Ashworth, 2004).

m. Combining fluorescence and other imaging modalities (e.g., transmitted light imaging).

Although advancements in fluorescence imaging technology coupled with the availability of a multitude of vital fluorescent probes have combined to make fluorescence the method of choice for most high resolution studies of living cells, it is sometimes advantageous to combine fluorescence imaging with other imaging modalities. For example, differential interference contrast (DIC) microscopy can be used in conjunction with scanning laser confocal microscopy to simultaneously monitor the whole cell in DIC mode while imaging the phagocytic uptake of fluorescent microspheres (Hook & Odeyvale, 1989) or the distribution of fluorescently-tagged proteins and molecules (e.g., Adams et al., 1998) within these cells.

Although it is difficult to perform DIC and epi-fluorescence imaging both simultaneously and optimally in widefield microscopy, it is somewhat easier to ensure that the fluorescence signal is not subjected to the light loss that occurs in the analyzer used as part of the DIC system if one uses a single-beam confocal. Thus, the DIC image can be collected from a fluorescently labeled specimen using transmitted light that would otherwise be wasted. Recently, differential phase contrast (DPC) has been implemented in a scanning laser microscope system (Amos et al., 2003), and this may offer additional capabilities where DIC optics are unsuitable. Notably for live-cell imaging, DPC reportedly needs 20 times less laser power at the specimen than DIC.

GENERAL CONSIDERATIONS FOR CONFOCAL MICROSCOPY OF LIVING CELLS

What factors must be considered when performing a live-cell confocal imaging experiment or observation? The major factors are to (1) label the preparation in order to clearly visualize the biological component of interest, (2) maintain the preparation in a condition that will support normal cell or tissue health, and (3) image the specimen with sufficient spatial and temporal resolution in a way that does not perturb or compromise it. **Table 19.2** outlines several of the most important experimental considerations for live -cell imaging, including the most common problems and some potential solutions.

Maintenance of Living Cells and Tissue Preparations

In vitro preparations

Specimen maintenance is a very important part of any live imaging study and usually requires both mechanical ingenuity and insight into the biology of the cell or tissue under study. The specimen chamber must keep the cells or tissues healthy and functioning normally for the duration of the experiment while allowing access to the microscope objective. This can be particularly difficult when high-numerical-aperture (NA) oil- or water-immersion lenses are used. In many cases, there must also be a controlled and efficient way to introduce a reagent to perturb a particular cellular process. Other important factors are simplicity, reliability, and low cost. At any rate, it is advisable to monitor the conditions within the imaging chamber carefully. It may be helpful to use microprobes that can detect pH, O_2, and CO_2 (e.g., Lazar Research Laboratories, Inc., Los Angeles, CA).

The early closed perfusion chambers designed by Dvorak and Stotler (1971) and later by Vesely et al (1982) were inexpensive and permitted high-resolution transmitted light observation. They relied on an external heater that warmed the entire stage area for temperature control.

Setups for different cells vary widely. Mammalian cells probably pose the greatest problems. McKenna and Wang's article (1986) is a general introduction to the problems associated with keeping such cells alive and functioning on the microscope stage. This article discusses culture chamber design as well as strategies for controlling pH, osmolarity, and temperature. The authors describe their own chamber, in which temperature is controlled by heating the air in a box surrounding the stage area, and mention earlier

designs such as the resistively heated Lieden Culture System first described by Ince et al (1983) and later improved by Forsythe (1991).

Strange and Spring (1986) describe their setup for imaging renal tubule cells where temperature, pH, and CO_2 are controlled. They provide a detailed account of the problems of establishing laminar flow perfusion systems, temperature regulation, and maintenance of pH by CO_2 buffering. Somewhat later, Delbridge et al. (1990) describe a sophisticated, open-chamber superfusion system permitting programmed changes of media, precision control of media surface height, and temperature regulation between 4°C and 70°C using a Peltier device to control the perfusate temperature. Myrdal and Foster (1994) used a temperature-stabilized liquid passing through a small coil suspended in media filling a plastic NUNC chamber to provide temperature control for confocal observations of the penetration of fluorescent antibodies into solid tumor spheroids. An automatic system maintained fluid level and bathed the area in CO_2 but special precautions were required to prevent drift of the confocal focus plane during long time-lapse sequences. Methods for observing microglial cell movements in mammalian brain slices are described in detail in a later section of this chapter.

Chambers have even been built for the microscopic observation of cells as they are being either frozen or thawed in the presence of media that could be changed during the process (for instance, Walcerz and Diller, 1991). In this case, computer-controlled pumps deliver temperature-controlled nitrogen gas at between -120°C and 100°C to special ports connected to a temperature cell (-55°C to 60°C) that forms the upper boundary of the perfusion chamber. Other ports carry either the perfusate or a separate nucleating agent to the cell chamber itself.

More recently, a specialized *in vitro* cell culture system has been developed to maintain mammalian neuronal cells for over a year (Potter and DeMarse, 2001)!

There are several companies that provide ready-made microscope stage chambers, temperature-control units, automated perfusion systems, and a variety of related accessories. These are summarized in Table **19.3**.

In vivo preparations

The ultimate goal of many research programs is to understand the normal (or abnormal) structure and function of molecules, cells, and tissues *in vivo*, that is, in the living organism functioning within its native environment (Megason & Fraser, 2003). There has been some remarkable progress recently on extending high resolution confocal and MP imaging in this direction, especially in preparations that are essentially translucent Several model organisms, including zebrafish (Cooper et al., 1999), frog (Fraser & O'Rourke, 1990; Robb & Wylie, 1999), fruit fly (Paddock, 2002), leech (Baker et al., 2003), and worm (Crittenden & Kimble, 1999), have emerged as excellent preparations for cellular and molecular imaging studies spanning a variety of biological questions. As an example, studies in the zebrafish have been carried out on the structural development of vasculature (Lawson & Weinstein, 2002; Isogai et al., 2003), cell

division (Gong et al., 2004; Das et al., 2003), neuronal migration (Koster & Fraser, 2001), axonal pathfinding (Dynes & Ngai, 1998), synapse formation (Jontes et al., 2000; Niell et al., 2004), and synaptic plasticity (Gleason et al., 2003), to name a few. Physiological studies in zebrafish have included, for example, imaging intracellular calcium during gastrulation (Gilland et al., 1999), in the intact spinal cord (O'Malley et al., 1996; Gahtan et al., 2002), and in brain (Brustein et al, 2003). Each of these biological preparations embodies its own unique set of specimen mounting and maintenance challenges. Indeed, it is sometimes necessary to anesthetize the preparation to prevent it from crawling or swimming away during the imaging session!

Perhaps the most difficult conditions involve imaging in a living mammal, an undertaking for which the confocal or MP microscope enjoys the twin advantages of epi-illumination and optical sectioning that make it possible to view solid tissues without mechanical disruption. Confocal microscopy has long been an important tool for *in vivo* imaging of eye tissues non-invasively (Petran et al., 1986; Jester et al., 1991, 1992; Masters, 1992; Petroll et al., 1992, 1993; Poole et al., 1993). In terms of imaging interior tissues, early studies described methods for examining microcirculation of the brain cortex in anesthetized rats (Dirnagl et al., 1992) or changes in kidney tubules during ischemia (Andrews et al., 1991). . Confocal microscopy also has been used to image leukocyte-endothelium interactions during infections through closed cranial windows (Lorenzl et al., 1993). More recently, MP has been used to image live mammalian brain tissues *in vivo*, either through a cranial window (Svoboda et al., 1997; Trachtenberg et al., 2002), fiber optic coupled devices (Mehta et al., 2004), or directly through the intact but thinned skull (Yoder & Kleinfeld, 2002). Dual-channel MP imaging also has been used to image other tissues *in vivo*, including lymphoid organs (e.g., Miller et al., 2002). It is generally accepted that MP imaging is superior to single photon confocal for these *in vivo* imaging studies (Cahalan et al., 2002).

Fluorescent Probes

Except in those cases where an adequate image can be derived from either the backscattered-light signal or from autofluorescence, confocal microscopy of living cells is dependent on the properties and availability of suitable fluorescent probes. In addition to binding specifically to what one is interested in studying, the fluorescent probe should produce a strong signal and be both slow to bleach and nontoxic. Chapters 16 and 17 (this volume) discuss fluorescent dyes that have been used in published work with confocal microscopy in detail.

Many dyes are useful when introduced to the medium surrounding cells to be labeled. Some of the classic and most commonly used cell stains include DiI for labeling the plasma membrane (Honig and Hume, 1986; Baker & Reese, 1993), $DiOC_6(3)$ for labeling internal membranes (Terasaki et al., 1984), NBD-ceramide and bodipy-ceramide which label the Golgi apparatus (Pagano et al, 1991), rhodamine 123 which labels mitochondria (Johnson et al, 1980), potential sensitive dyes (Loew, 1993), and FM 1-43 (Betz et al, 1992) which is used to follow plasma membrane turnover and vesicular release. Fluorescent ion

indicators such as Fluo-3 (Minta et al, 1989) can either be microinjected or added to the media in a cell-permeant acetoxymethylester form that becomes trapped inside the cell after being cleaved by intracellular esterases. (See Chapter 42, this volume.)

Minimizing Photodynamic Damage

Once the cells are labeled and on the microscope, one is faced with the challenge of collecting data without compromising the cell or bleaching the label. In practice, the major problem is light-induced damage. Fluorescent molecules in their excited state react with molecular oxygen to produce free radicals that can then damage cellular components and compromise cell health (Dixit & Cyr, 2003).

In addition, several studies suggest that components of standard culture media might also contribute to light-induced adverse effects on cultured cells (see Siegel and Pritchett, 2000). Some early studies (Spierenburg et al., 1984; Zigler et al., 1985; Lepe-Zuniga et al., 1987; Zieger et al., 1991) indicated a phototoxic effect of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) containing media on cells under some circumstances. It seems possible that this effect might be more directly related to inadequate levels of bicarbonate (Cechin et al., 2002). Other studies suggest that riboflavin/vitamin B2 (Zigler et al., 1985; Lucius et al., 1998) and the essential amino acid tryptophan (Griffin et al., 1981; Silva et al., 1991; Silva and Godoy, 1994; Edwards et al., 1994) may mediate phototoxic effects. Whether these effects occur under typical confocal imaging conditions is unknown, but many of the photoeffects are eliminated by anti-oxidants, so it seems advisable to maintain anti-oxidants (and some bicarbonate, as well) in the specimen chamber (see below) and to use photons with great efficiency.

Improving Photon Efficiency

There are several strategies to minimize the amount of excitation light required to collect data (see Chapters 2 and 12, this volume, for more details). Briefly, higher-NA objective lenses collect more of the fluorescent emission. For a given lens, there is also a theoretical optimal setting of the zoom magnification that best matches the resolution required to the allowable dose (see Chapters 4, this volume). When the focus plane is more than 10μm from the coverslip, water-immersion lenses should be used to avoid the signal loss caused by spherical aberration when using an oil lens (see Chapters 7 and 20, this volume).

Another way to reduce light damage is to minimize the duration of the light exposure during the experimental setup. For instance, one should try to focus as rapidly as possible and turn off the light source as soon as the focus range has been chosen. In addition, in single beam scanning systems, make sure that your scanner is set up to blank the laser beam during scan retrace. Otherwise, areas on both sides of the imaged area will receive a very high light exposure as the beam slows down to change direction.

Finally, photon efficiency can be maximized by using the best mirrors, the correct pinhole size for the resolution required (in x, y, and z), and photodetectors that yield the highest quantum efficiency at the wavelength of the signal.

Anti-oxidants

As noted above, one can also reduce photodynamic damage by adding anti-oxidants to the medium. Oxyrase (Oxyrase Inc., http://www.oxyrase.com) is an enzyme additive used to deplete oxygen in order to grow anaerobic bacteria. It has been used at 0.3 unit/ml to reduce photodynamic damage during observations of mitosis (Waterman-Storer et al., 1993). Another approach is to include ascorbic acid in the medium. This reducing agent is typically used at 0.1-1.0 mg/ml but has been used at up to 3 mg/ml. A recent confocal study of calcium transients in isolated chrondrocytes reported a relationship between laser intensity and the frequency of Ca^{2+} oscillations and cell viability: Ca^{2+} events were more frequent and cell viability was decreased with higher laser intensity (Knight et al., 2003). Treatment with ascorbic acid reduced the Ca^{2+} events and improved cell viability (see also Chapters 16 and 17).

The On-line Confocal Community

Confocal microscopy of living cells is an area of active research where individuals are constantly developing new techniques and approaches. One way to keep up with current practice is to join about 1,600 others who subscribe to the Confocal e-mail listserver. This can be done by registering at the listserver website, located at http://listserv.buffalo.edu/archives/confocal.html. You will then begin to receive messages from other microscopists. Recent topics have included discussions on such diverse issues as autofluorescence problems, glass-bottomed culture chambers, damage to live cells during FRAP experiments, and announcements of confocal workshops. The listserver also has an extensive, searchable archive dating back to 1991, and this is freely accessible.

A Convenient Test Specimen

Knebel et al. (1990) showed that onion epithelium (*Allium cepa*) is a simple preparation that can be used as a convenient test specimen for confocal microscopy of living cells. Figure **19.1** shows how to prepare onion epithelium. First, a small square of a layer is cut out using a razor blade. A forceps is used to peel off the thin epithelium on the inner surface of the onion layer. The epithelium is then put onto a microscope slide, covered with a drop or two of staining solution containing $DiOC_6(3)$, a marker of mitochondria and endoplasmic reticulum, and coverslipped. The stock solution of $DiOC_6(3)$ (0.5 mg/ml in ethanol) can be kept indefinitely if protected from light in a scintillation vial. The staining solution is a 1:1000 dilution in water on the day of the experiment. The center of these cells is usually occupied by a large vacuole, and the ER and mitochondria are located in a thin cytoplasmic region near the plasma membrane. Motion of the ER is relatively quick and easily detected in consecutive 1-sec scans.

SPECIFIC EXAMPLE I: VISUALIZING CHROMATIN DYNAMICS USING VERY LOW LIGHT LEVELS.

It is clear from the discussion above that microscopy of living cells has become an technique of major importance in cell biology: it can be used to tell us *where* molecules are located, *when* they become localized, *how fast* they are moving, with which molecules they are *interacting*, and how long they stay *attached* to these molecules. All these properties can be observed in the natural environment of the living cell. The major limiting factor in live-cell imaging is phototoxic effect of light used for the observation of the cell. Here we will address some practical issues of phototoxicity based on our experience in imaging chromatin dynamics in living cells (Manders et al., 1996; 1999; 2003; Moné et al., 2004; Verschure et al., 1999).

1 Phototoxicity

A large number of photochemical reactions are responsible for the phototoxic effect of light. Light can be absorbed by cellular components and induce chemical alterations in the molecular structure. For example, UV-light is absorbed by DNA (absorption peak at 280 nm), directly inducing DNA-damage. Here we assume that, working with visible light, the direct photodamage is negligible. In fluorescently labelled cells, the main source of photodamage is the production of reactive oxygen species (ROS) including singlet oxygen ($^1O_2$), superoxide ($\cdot O_2^-$), hydroxyl radical (HO·), and various peroxides. These activated oxygen species react with a large variety of easily oxidizable cellular components, such as proteins, nucleic acids, and membrane lipids. Singlet oxygen is responsible for much of the physiological damage caused by reactive oxygen species. For the production of singlet oxygen the fluorescent label acts as a photosynthesizer in a photochemical reaction where dioxygen ($^3O_2$) converts into singlet oxygen ($^1O_2$). Singlet oxygen mainly modifies nucleic acid through selective (oxidative) reaction with deoxyguanosine into 8-oxo-7,8-dihydro-2'-deoxyguanonine. Proteins and lipids also will be damaged by ROS. Phototoxicity likely depends on several variables:

1) **The photochemical properties of the fluorescent molecule.**
   Some molecules induce more phototoxicity than others, depending on the lifetime of their triplet-state. For photodynamic therapy (PDT) dedicated molecules called photosensitizers, have been designed in order to induce a maximum damage in tissue for the treatment of cancer (e.g. halogenated fluorescein is much more toxic than fluorescein). Another property that influences the phototoxicity of a molecule is the local environment of the molecule. The active fluorophore of a GFP molecule is positioned on the inside of the protein, within the barrel structure (the "β-can"). Probably this hydrophobic, protein-environment contributes to the relatively low phototoxicity of GFP compared with 'naked' fluorophores such as fluorescein or rhodamine.
2) **The subcellular location of the fluorescent molecule.**
   When fluorescent molecules are situated close to DNA, the damaging effect of singlet oxygen is more pronounced. Despite several DNA-repair mechanisms, the cell will not continue its cell cycle (arrest)

and may even die if there is too much DNA damage. Therefore, fluorophores in the cytoplasm seem to induce less phototoxicity than fluorophores in the nucleus.

3) **The concentration of fluorophore.**

   It is clear that there is a relationship between the local concentration of fluorophore and the level of phototoxicity. We assume a linear relationship between fluorophore concentration and toxicity, although this has not been assessed directly and is complicated by the fact that if there is more dye, one need use less excitation.

4) **The excitation intensity.**

   Fluorescent cells in a dark incubator are quite happy for weeks as long they are maintained in the dark. As the word 'phototoxicity' implies, photons are needed to induce toxicity in fluorescently labeled specimen. We usually assume a linear relationship between excitation light dose and toxicity, although the temporal regimen of the excitation may be important to how cells handle the accumulation of phototoxic biproducts. Phototoxicity is dependent on the wavelength of light in the sense that the wavelength of the 'toxic' excitation light matches the excitation curve of the fluorophore. In other words, it is the excited fluorophore that is toxic. Koenig also found that, with 2-photon excitation, the damage is proportional to the number of molecular excitations (See Chapter 38).

There is no clear evidence for differences in phototoxicity between green, red, or far-red fluorophores. In principle, excited Cy5 can be as toxic as excited FITC. However, the wavelength of excitation light can be a factor when imaging in thick specimen because stronger incident illumination is needed for comparable excitation of shorter wavelength fluorophores due to increase tissue scatter at shorter wavelengths.

2 Reduction of photo-toxicity

For many researchers, phototoxicity is a serious (and annoying!) limitation of their observations of living cells. When you do not look at a cell it is alive, but the moment you start to observe how it lives, it is killed by the light used to observe it. In experiments so far, we have succeeded in obtaining acceptable time series of living cells by carefully optimising all steps in the imaging process in an effort to achieve (i) maximal signal-to-noise ratio, (ii) maximal spatial and temporal resolution, and (iii) minimal phototoxic effects. Specifically, phototoxicity has been minimized by (i) using radical scavengers (e.g., trolox) in the culture medium, and (ii) using culture medium without phenol-red. **Most important of all, however, is minimizing the total excitation light dose**. The excitation light dose is the product of the light intensity and the exposure time. Decreasing either the excitation intensity or the excitation dose implies a loss of fluorescent signal. It is inevitable that a reduction of light dose puts a limitation on the S/N ratio and the spatial and temporal resolution. **The art of live-cell microscopy is finding the balance between image quality and cell vitality.**

3 Improving image quality in low-dose microscopy

Figure **19.2A** shows a single time frame projection from a 3D time-series of a HeLa cell expressing the fluorescent histone fusion protein, H2B-GFP. In the time series shown in Fig. 19.2B, the cell is in late telophase at the start of the imaging and proceeds into interphase during the movie. This movie shows data from a study on the dynamics of chromatin during decondensation (Manders et al. 2003)[1]. In these experiments the excitation light intensity was kept below 150 nW[2] and the total exposure time of a cell that was 3D-imaged for three hours was not more than 70 sec. Under these conditions the total light dose was approximately10 J cm^{-2}. In experiments where we used a higher dose of light we observed considerable phototoxic effects, i.e., cell cycle arrest and cell death.

Reducing the total light dose during an experiment requires that the number of 3D images in the sequence (temporal sampling rate) is low. Because of this limited sampling rate, live-cell movies are usually under-sampled in time according to the Nyquist criterion. As a result, such movies often show cells that nervously move from one place to another and sometimes suddenly rotate. We have applied an image processing procedure to correct for all the movements (translation and rotation) of the cell. For each 3D image of the time sequence, a translation and rotation transform vector was calculated in order to obtain a best fit with the previous image in the sequence. After a series of such transformations, a new movie was produced showing a stable cell that does not move or rotate. Only internal movements are visible. After this correction procedure, we applied a simple Gaussian spatial filter to reduce noise in the image (Fig. 19.2C,D). We also applied a temporal filter by adding to each voxel of the 3D image at each time-point the value for that voxel in the previous and subsequent image multiplied by an intensity factor of 0.5. Our experience is that temporal filtering makes the movie easier to interpret.

4 Low dose imaging conclusion

The success of live -cell microscopy is very much dependant on minimizing or avoiding any toxic effect of light on the biological system under observation. A certain dose of light may induce serious DNA damage that may arrest the cell cycle, whereas the diffusion coefficient of a certain protein is not influenced at all at the same dose. In the experimental example shown here (Fig. 2B) we used only 150 nW of incident beam power. This dose was found to be phototoxic in other experiments using fluorescein instead of GFP, and it was found necessary to drop the laser power to 50 nW (Figure 19.2E). These power levels are far lower than (i.e., <1% of) those commonly used in confocal microscopy, a circumstance facilitated at least in part by the fact that the chromosomes are quite heavily stained.

Our collective experiences indicate that the effect of phototoxicity depends on the cell type, the stage of the cell cycle, the fluorophore, the observed biological process, and many other experimental conditions. We

[1] Both movies will be on the Springer WWW site associated with this book.
[2] Power levels were measured using a photometer sensor that was oil-coupled to the specimen side of an oil-coupled coverslip.

conclude that there is no general guideline for the maximum allowable laser power: it must be assessed empirically for each experimental condition. As a general rule, however, images of living cells are almost always more noisy than images of fixed preparations because the incident illumination intensity needs to be kept to a minimum to maintain cell viability. A noisy image in which one can see what is absolutely essential is of more use than a "better" image of a damaged cell. Keep in mind that not all types of damage are equally easy to detect. Damage may disturb (or create!) a monitored process, it may interfere with cell division, or it may cause the cell to bleb and pop! We recommend that that the experimentalist BEGIN by assuming that "to observe is to disturb." Measure the power level coming out of the objective. Try your experiment again using twice the power, and again using half the power. Make sure that you can explain any 'behavioral' differences between these runs.

We show here that some simple image processing (deconvolution) can help facilitate the analysis of live-cell imaging data by reducing noise and improving the point-to-point coherence when viewing a time-series of images. Note the reduction in noise level between Fig. 19.2C and 19.2D .

SPECIFIC EXAMPLE II: MULTI-DIMENSIONAL IMAGING OF MICROGLIAL CELL BEHAVIORS IN LIVE RODENT BRAIN SLICES

The example above serves to illustrate that confocal microscopy is an important tool for studying dynamic subcellular processes in live, isolated cells. Many biologists are also interested in understanding dynamic structural and functional aspects of cells within the context of a natural tissue environment. As noted above, confocal and multiphoton imaging have been applied to intact, normally functioning systems such as the eye, skin, or kidney. Some recent studies have even extended these observations beyond superficial tissues to deep tissues of the brain (e.g., Mizrahi et al., 2004).

However, some tissues are much less accessible, or it may be of interest to be able to experimentally perturb or control the system under study. For these purposes, the *in vitro* tissue slice has been an important experimental preparation (e.g., Gähwiler et al., 1997). Smith et al. (1990) were among the first to show the feasibility of imaging the structure and physiology of living mammalian brain tissue slices at high resolution using fluorescence confocal microscopy. Since then many confocal studies of both structural and physiological dynamics of cells in tissue slices have appeared, and it seems that interest in imaging *in vitro* tissues is continuing to grow. We will address here some of the most common problems, challenges, and limitations inherent in confocal studies of live tissue slices. These points will be illustrated by drawing from our own time-lapse studies in live tissue slices of developing mammalian central nervous system (CNS) (Dailey and Smith, 1993, 1994, 1996; Dailey et al., 1994; Dailey and Waite, 1999; Marrs et al., 2001; Stence et al., 2001; Grossmann et al., 2002; Petersen and Dailey, 2004; Benediktsson et al., 2005).

Some of the major problems encountered when imaging fluorescently labeled cells in live tissue slices are:

- **Attaining a suitable level and specificity of staining.**

- **Maintaining cell/tissue health:** pH, temperature, oxygen, etc.
- **Keeping cells in focus:** can be an immense problem when following cells over long periods of time:
    - movement of the microscope stage, especially when stage heaters are used.
    - movement of the tissue: apparent movement that is really caused by movement of the focal plane within the specimen; natural movement of whole organisms or those caused by heartbeat, etc.
    - movement of cells within the tissue, e.g., cell migration, extension/retraction of cell processes.
    - movement related to experimental procedures, e.g., stimulus-induced osmotic changes.
- **Attaining a useful image with a high signal-to-noise ratio of cells deep within tissue:**
    - imaging away from damaged tissue surfaces.
    - light scatter by the tissue.
    - the problem of spherical aberration.
- **Handling data:** viewing, storing, retrieving, and analyzing 4D data sets:
    - short-term: monitoring experiments on the fly; adjusting focus.
    - long-term: accessibility and security of archived data.

We have been exploring the dynamic behavior of a type of brain cell, termed microglia, following brain tissue injury. These cells undergo a dramatic transformation ("activation") from a resting, ramified form to an amoeboid-like form within a few hours after traumatic tissue injury. Activation of microglia is triggered by signals from injured cells (including neurons), and this mobilizes microglia to engage neighboring dead and dying cells. Naturally, these events are best studied in the context of a complex tissue environment containing the native arrangement of tissue components; thus time-lapse confocal microscopy is well suited to examine these events. The general approach we have taken is to label the cell surface of microglia with fluorescent probes and, subsequently, to follow the dynamic movements of these cells, as well as their interactions with other labeled cells, within live tissue slices continuously over periods of time up to 28 hr (Dailey and Waite, 1999; Stence et al., 2001; Grossmann et al., 2002; Petersen and Dailey, 2004).

Preparation of CNS Tissue Slices

A useful method of preparing and maintaining live brain tissue slices for microscopy is based on the organotypic (roller-tube) culture technique of Gähwiler (1984) or the static filter culture technique of Stoppini et al. (1991). Briefly, these techniques involve rapidly removing the tissue of interest (in this case, neonatal rat or mouse hippocampus), then slicing the tissue with a manual tissue chopper (Stoelting, Chicago, IL) at a thickness of 300-400 μm. Others have used a vibratome or custom-built instruments akin to an egg slicer. In the case of the roller tube technique, the tissue slices are secured to an alcohol-cleaned coverslip (11 x 22 mm) with a mixture of chicken plasma (10 μl; Cocalico) and bovine thrombin (10 μl; Sigma). Collagen gels (Vitrogen; CellTrix; O'Rourke et al., 1992) and Cell-Tak (BioPolymers Inc.; Barber et al., 1993) have also been used successfully to attach slices. In the case of the plasma clot, the slices are

adherent within about 10 min, at which point the coverslips are placed in a test tube with 1 ml of HEPES-buffered culture media containing 25% serum. The tubes are kept in a warm box (37°C) and rotated at 12 rph in a roller drum tilted at 5° to the horizontal. In the case of the static filter cultures, brain slices are placed on porous cell culture inserts in 6-well plates containing ~1ml of bicarbonate-buffered culture media per well. The filter cultures are maintained at 36°C in a 5% CO_2 incubator. In either case, these "organotypic" culture methods provide a means for maintaining tissue slices in vitro for up to several weeks (Gähwiler et al, 1997).

Fluorescent Staining

Microglia. Often it is most useful to label only a small percentage of the total number of cells within a tissue volume, and in certain cases it is desirable to label only an identified subset of cells. Vital fluorescent probes must be non-toxic and resistant to photobleaching. In the case of microglia, there are several commercially available fluorescent conjugates (FITC, Alexa Fluor–488, –568, or –647) of a highly selective, non-toxic lectin (IB_4) derived from *Griffonia simplicifolia* seeds (Sigma; Molecular Probes). This lectin has an exclusive affinity for α-D-galactosyl sugar residues on glycoproteins and glycolipids (Wood et al., 1979). In mammalian brain tissues, IB_4 labels only microglia and endothelial cells lining blood vessels and capillaries (Streit and Kreutzberg, 1987). The simultaneous labeling of microglial cell populations and blood vessels has revealed novel, dynamic interactions between these structures (Grossmann et al., 2002). Incubation of brain slices or slice cultures for 1 hr in IB_4-containing medium (5μg/ml) is sufficient for robust labeling of microglia up to ~50μm deep within tissues (Kurpius and Dailey, 2005).

Nuclei of live or dead cells. One of a variety of fluorescent DNA-binding dyes is used to label live or dead cell nuclei in brain tissue slices (Dailey and Waite, 1999; Petersen and Dailey, 2004). To visualize the nuclei of live cells, we use one of the membrane-permeant dyes that have spectra in the far-red, SYTO59 (abs/em: 622/645nm) or SYTO61 (620/647nm). For labeling nuclei of dead cells, we use one of the membrane impermeant dyes, Sytox Green (504/523nm), Sytox Orange (547/570nm), or To-Pro-3 (642/661nm) (all from Molecular Probes). All DNA-binding dyes are applied for 10-20 min (1:10,000). These dyes are used in combination with fluorescently tagged IB_4 to image microglial behaviors in relation to other cells.

Maintaining Tissue Health on the Microscope Stage

Image data obtained from compromised tissue is useless at best, and deceiving at worst. For example, CNS slice physiologists have long known that oxygen deprivation can have severe effects on synaptic activity, although CNS tissues from developing animals seem to have a fairly high resistance to hypoxia (Dunwiddie, 1981).

It is not always easy to assess the health of living tissue on the microscope stage, but in the case of dynamic processes such as cell division or cell migration, one would at least expect that the cells perform

these activities at rates near that expected based on other methods of determination. Also, one should become suspicious if the rate of activity consistently increases or decreases over the imaging session. For example, exposure of fluorescently labeled axons to high light levels can reduce the rate of extension or cause retraction. In contrast, high light levels can produce a long-lasting increase in the frequency of Ca^{2+} spikes in Fluo-3-labeled astrocytes in cultured brain slices. In many cases, there will not be a useful benchmark for determining phototoxic effects, but consistent changes during imaging will serve to warn the concerned microscopist. It may be worth sacrificing a few well-labeled preps to determine if different imaging protocols, such as lower light levels or longer time intervals between images, significantly alter the biological activity under study.

Requirements for maintaining healthy tissue during imaging dictate specimen chamber design. A closed specimen chamber has the advantage of preventing evaporation during long experiments and stabilizing temperature fluctuations caused by this evaporation. We found that microglia in tissue slices maintained in a closed chamber (volume ~1 ml) with HEPES-buffered culture medium remain viable and vigorous for about 6 hr, after which point the chamber medium acidifies and cell motility declines. However, when the old chamber medium is exchanged with fresh medium, the cells jump back to life again. This crude method of periodic medium exchange has supported continuous observation of DiI-labeled migrating cells in tissue slices on the microscope stage for as long as 45 hr (O'Rourke et al, 1992). However, when using this approach, one runs the risk of mechanically disturbing the chamber or inducing a temperature change and thereby causing a jump in focus.

A more elegant method for medium exchange and introduction of reagents involves continuous superfusion. A variety of perfusion chambers with either open or closed configurations are available (see **Table 19.3**). Sometimes it is necessary to design and construct very sophisticated temperature and fluid-level control systems (e.g., Delbridge et al, 1990, or Walcerz and Diller, 1991). Such chambers permit very rapid exchange of medium (seconds rather than minutes), which is necessary for physiological experiments requiring high time resolution. There are also now commercially available, programmable, automated perfusion systems that permit rapid switching between one of several perfusion channels (see **Table 19.3**). Some experimental conditions require only relatively simple, low-cost chambers and perfusion systems, such as the one depicted in Fig. **19.3** (see also Dailey et al., 2005). We have used such a design to continuously superfuse tissue slices on the microscope stage for many hours. The tissues seem to remain healthy for at least 20 hr when perfused (10-20 ml/hr) with either the culture medium (see Dailey et al., 1994) or normal saline, both of which are buffered with 25 mM HEPES.

Specimen heating is essential for many experiments, but this can induce an agonizing battle with focus stability (see below) as the chamber and stage components heat up. Because there is always a time lag between when the sensor of the temperature control detects that the temperature is too high (or low) and the time that the heater is able to warm the whole stage, the actual temperature of most stage heater is always slowly oscillating, a fact that causes the focus plane to shift in a periodic manner. A sufficient period of

preheating can sometimes reduce this problem. Another approach is to use a modified hair dryer to blow warm air onto both the chamber and the stage (see Dailey et al., 2005) or an egg-incubator heater to heat all the air in an insulated box surrounding the entire microscope (Potter, 2004).

It is also important to monitor the temperature of the perfusing medium very near to the specimen. A low-cost microprocessor temperature controller that reduces fluctuations in the heating/cooling cycle can be obtained from Omega Engineering (Stamford, CT).

Imaging Methods

It should by now be evident that a primary concern when imaging living cells is photon collection efficiency. This is especially true when imaging dynamic processes, such as cell migration, over long periods of time. Higher collection efficiency will afford effectively lower excitation light levels, thus permitting more frequent sampling or observations of longer duration.

We have been using commercially available laser scanning confocal systems (Leica TCS NT and Leica SP2 AOBS). For illumination, the microscopes are equipped with multiple lasers. Both systems offer simultaneous excitation and detection in more than one epi-fluorescent channel. In addition, the SP2 AOBS system offers increased flexibility by providing continuously variable wavelength selection in the emission pathway. This permits customizing the spectral detection to maximize throughput in separate channels. The Leica objectives that we find most useful are a dry 20x/0.75 PlanApo lens, and a 63x/1.2 water-immersion PlanApo objective lens.

Imaging Deep within Tissue

Often the goal of studies in tissue slices is to examine biological processes within a cellular environment that approximates that found *in situ*. In the case of tissue slices, it is usually desirable to image as far from cut tissue surfaces as possible to avoid artifacts associated with tissue damage. However, the cut surfaces of developing CNS tissue slice cultures contain a plethora of astrocytes, activated microglia, and a mat of growing neuronal processes. Time-lapse imaging of these regions provides striking footage of glial cell movements, proliferation, and phagocytosis (Stence et al., 2001; Petersen and Dailey, 2004).

With oil-immersion lenses, useful fluorescence images seem to be limited to a depth of 50-75 μm or so into the tissue. Imaging deeper (> 50 μm) within tissue can suffer from several factors, including:

- Weak staining of cells due to poor dye penetration
- Light scatter by the overlying tissue components
- Spherical aberration

The first problem can be overcome if the dye can be injected into the tissue with a minimum of disruption, or if tissues can be harvested from transgenic animals expressing fluorescent proteins in subsets of cells (e.g., Zuo et al., 2004). Light scatter by the tissue can be minimized by using longer-wavelength

dyes (see Chapters 28 and 37, this volume). Indeed, we find that a long-wavelength (Alexa Fluor-647; Molecular Probes) conjugate of IB4 noticeably improves visibility of labeled cells in deeper portions of the tissue slice. Imaging at longer wavelengths may also reduce phototoxic effects since the light is of lower energy. Finally, the problem of spherical aberration can be improved by using water-immersion objective lenses or the recently introduced automatic spherical aberration corrector (Intelligent Imaging Innovations, Denver, Colorado, and also Chapters 1, 7, and 20, this volume).

Keeping Cells in Focus

The optical sectioning capability of the confocal microscope can be simultaneously a blessing and a curse. On the one hand, thin optical sections reduce out-of-focus flare and improve resolution. However, with such a shallow depth of focus, even very small changes in the position of the objective lens relative to the object of interest within the specimen can ruin an otherwise perfect experiment. This is a particular problem when imaging thin, tortuous structures such as axons or dendritic spines within neural tissue. A moving focal plane can, for example, give one the erroneous impression of dendritic spine extension or retraction. This problem is compounded when imaging cells and cell processes that are in fact actively moving within the tissue.

One obvious approach is to image the cells in four dimensions (3D x time). This can absorb some changes in tissue and stage movement as well as help track cells that move from one focal plane to another. In addition, our strategy has been to image with the detector pinhole aperture substantially open, corresponding to a pinhole size roughly 4 Airy units in size. Although this reduces the axial resolution slightly, it has the dual advantage of achieving a higher signal-to-noise ratio at a given illumination intensity as well as thickening the optical section. On our microscope systems, the open pinhole configuration gives an apparent optical section thickness of about 3 μm when using a 20x NA 0.7 objective. Thus, for each time point, we collect about 15 images at axial step intervals of ~2 μm. The guiding principle here is to space the image planes in the axial dimension so as to maximize the volume of tissue imaged but not lose continuity between individual optical-section images.

When these image stacks are collected at ~5-min intervals at power levels of ~50-75 μW (back aperture of objective), IB_4-labeled cells do not appear to suffer phototoxic effects and can be imaged continuously for over 20 hr. Image stacks can be recombined later using a maximum brightness operation. Unfortunately, even when z-axis stacks of images are collected, tissue movements can be so severe as to necessitate a continuous "tweaking" of the focus. Thus, it is helpful to monitor image features on the screen (by making fiduciary marks on an acetate sheet taped to the monitor screen), or to store the data in such a way that they are quickly accessible and can be reviewed on the fly to make corrective focus adjustments.

Ideally, one would like an automated means of maintaining the desired plane of focus, especially for long imaging sessions. Although there are several autofocus methods that work for simple specimens (e.g.,

Firestone et al., 1991), imaging structures in 3D tissue presents a significant challenge because there is no single image plane on which to calculate focus.

Handling the Data

Imaging tissue in 3D over time solves some problems but generates others. In particular, how does one deal with the Gigabytes of image data that are often obtained in a single experiment? Fortunately, improvements in desktop computer performance and storage capacity, coupled with low cost, make this much less of a problem than it used to be. Desktop workstations now contain hard drives with very large storage capacities (hundreds of Gbytes), and archiving methods and media are readily accessible and inexpensive. We typically store newly acquired data on a network server for image processing (such as spatial filtering or deconvolution), and analysis, then archive the data onto CDs or DVDs. This is a technology that is sure to continue rapid advancement, making it easier to store and access image data. At the end of the time-lapse experiment, the z-axis image stacks are combined in a variety of ways for viewing. For time-lapse studies, it is generally most useful to produce a set of "extended focus" images for viewing time-points in rapid succession. Depending on the file format, these image series can be viewed in one of a number of image viewers, including Scion Image or ImageJ (freely available from Scion Corp.). To create the projection images, we use a maximum brightness operation running in a custom-written Pascal macro to construct a 2D representation of the 3D data set (Stence et al., 2001). When the axial step interval is appropriate (see above), portions of single cells that pass through the various focal planes appear contiguous. Alternatively, the image stacks can be reassembled into a set of red-green 3D stereo images. Such images, when played in rapid succession, provide 3D-depth information as well as time information in thick tissue samples. If more than one fluorescent channel is used, they can be combined to create multicolor images.

Results

The ability to collect 3D image data sets over long periods of time and at relatively short time intervals has revealed new information on the dynamics of microglial cell activation, migration, and cell-cell interactions in live mammalian brain tissues. Here we offer some examples of the kind of data that can be obtained, along with some possible modes of data analysis.

In Fig. **19.4**, we show an example of a one-channel, single focal plane confocal time-lapse observation. Several IB4-labeled microglial cells in a live, "roller tube" slice culture were repeatedly imaged at intervals of a few minutes to reveal movements of microglial cells over time. These movements can be quantified using automated edge detection (Fig. **19.4a**). Computer analysis using 2D Dynamic Image Analysis Software (DIAS; Soll, 1995) shows differences in motile behavior of three cells, as determined by tracing the cell perimeter (Fig. **19.4b**). Cell motility movements can be displayed as regions of protrusion (green) or retraction (red) by comparing cell shape over successive time-points (Fig. **19.4c**). Many

parameters of cell shape and movement can be quantified easily using DIAS (Soll, 1995, 1999; Heid et al., 2002).

Dual-channel, time-lapse confocal imaging of a specimen labeled with more than one fluorophore can provide information on the dynamic relationship between cell components or diverse cell types. In Fig. **19.5**, we show a two-channel time-lapse series of IB4-labeled microglia and cell nuclei. Images from each channel (green and red) were collected simultaneously using a two-detector system. The nuclear marker (SYTO 61) labels all cells, including microglia. Time-lapse imaging shows the location of nuclei within migrating cells (Fig. **19.5a**), and DIAS analysis can be used to plot and analyze the movements of nuclei (Fig. **19.5b**).

Dual-channel, 3D time-lapse confocal imaging can yield information on the relative movement and location of structures labeled with two or more fluorophores within a thick tissue specimen. In the example shown in Fig. **19.6**, the movements of IB4-labeled microglia are observed in the context of dead cell nuclei labeled with To-Pro-3, a membrane-impermeant DNA binding dye. The stereo images reveal the movements of microglia on the surface of the tissue slice as well as deep within the tissue. Such information is being used to study differences in the behaviors and cell-cell interactions of microglia in a brain tissue environment. Note, for example, the rapid microglial movement and phagocytosis of a dead cell nucleus in the time-lapse series (Fig. **19.6**).

In summary, the 4D and 5D imaging methods outlined here capture more of the events occurring within the tissue and also provide the researcher with assurance that apparent changes in the length of even the smallest branches and fine filopodia are not due to the structures moving in and out of the focal plane. They also provide a means for exploring the dynamic interactions between different molecules, cells, and cell types within thick biological specimen.

Conclusions

- Imaging of living specimens at a high signal-to-noise ratio can be achieved with very low levels of incident illumination.
- Imaging with a fairly large pinhole strikes a balance between image quality and focus problems during long-term observation of live tissue.
- 4D time-lapse imaging is useful for following moving cells and fine cellular processes within complex tissues. Under optimal conditions, 3D data can be collected over long periods of time (> 20 hr) at relatively short time intervals (~5 min). Multi-channel (5D) imaging can provide information on the dynamic relationship of different cell or tissue components. Future developments should address constraints on high-resolution imaging deep (> 50 μm) within tissue. Improvements will likely be achieved by using water-immersion lenses and external automatic spherical aberration correctors (see Chapter 20) and by employing longer-wavelength dyes to reduce light scatter by the tissue and to minimize phototoxic effects.

FUTURE DIRECTIONS

For confocal microscopy of living cells, the most important characteristic of the instrument is its efficiency in collecting and detecting the fluorescence emission light from the specimen (Chapter 2 this volume). Any improvement in this efficiency reduces the amount of light damage and allows the gathering of more data. The increased data can either be in the form of more images, images with less statistical noise, or images obtained with greater spatial or temporal resolution. Newer models of existing commercial confocal microscopes have substantially improved photon efficiency. In addition, there have been technological improvements in the ability to separate the excitation and fluorescence emission of fluorophores, providing greater flexibility for multi-channel imaging and quantitative image analysis in live cells and tissues. Finally, the advantages of either Gaussian-filtering 2D data or deconvolving 3D data to reduce the effects of Poisson noise are now widely appreciated. Routine application of this approach can reduce the light load to the specimen by a factor of from 10 to 100 while still producing images with the same apparent resolution and signal-to-noise.

Technological and conceptual advancements are also likely to push the spatial and temporal resolution and other modes of fluorescence microscopy (e.g., Gustafsson, 1999; Hell et al., 2004 and Chapters 13, 30 and 31). Some of these approaches (e.g., 4Pi-microscopy) look promising for live cells (Gugel et al., 2004), but their potential for widespread use in biological applications has yet to be established, and there are limitations on the sample thickness (Gustafsson, 1999 and Chapter 21). In addition, higher resolution implies smaller pixels and therefore more photons/μm^2 and more bleaching and toxicity. Undoubtedly, there will be more improvements and wider applications along these lines in the future.

Although it is difficult to predict the future of confocal microscopy of living cells, as confocal microscopy (and its richer cousin, multiphoton microscopy) are in all probability the optimal methods for studying the 3D structure of living cells, the future seems sure to be bright!

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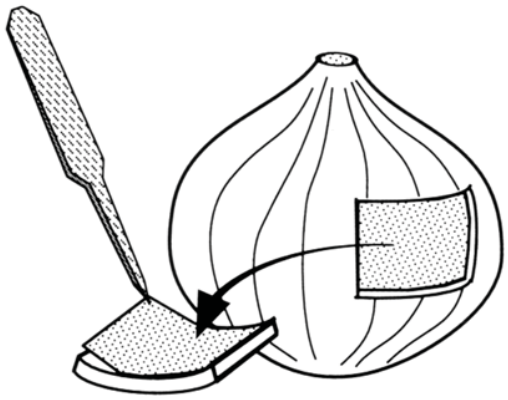

Fig. 19.1 – A convenient test specimen for confocal microscopy of cells in a living tissue: onion epithelium. This drawing shows how to obtain onion epithelium. As described in the text, the epithelium is stained with $DiOC_6(3)$, and the ER and mitochondria within the cells provide a bright and motile specimen.

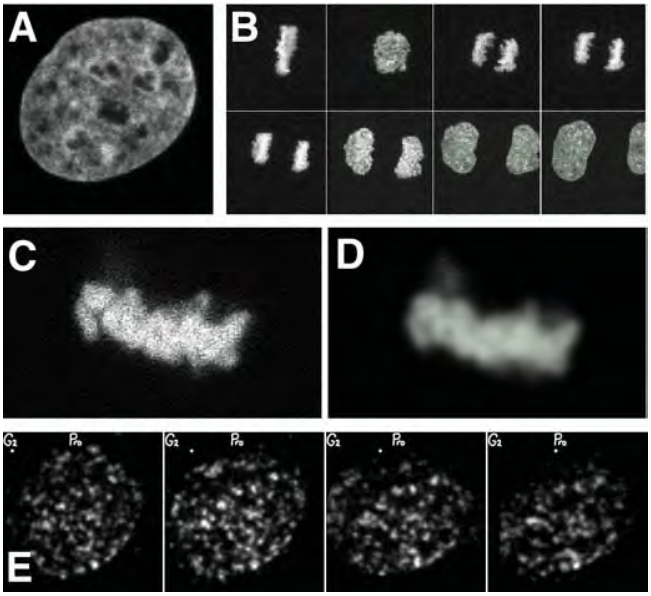

Fig. 19.2 – (A) A single time frame from a time-series of a HeLa cell expressing the fluorescent histone fusion protein, H2B-GFP. The cell is in late telophase. (B) A time-series of another cell shows how it proceeds into interphase during chromatin decondensation. In this experiment, the excitation light intensity was kept below 150 nW. Higher laser power induced cell cycle arrest. (C,D) Application of a simple Gaussian spatial filter reduces noise in the raw image (C) and improves image quality (D). (E) Time-series showing chromatin structure in a cell proceeding from G2 (left) to prophase (right). Note that the images were captured with a very low power dose (50nW). See the supplemental video movie at http\\. For details, see Manders et al., 2003.

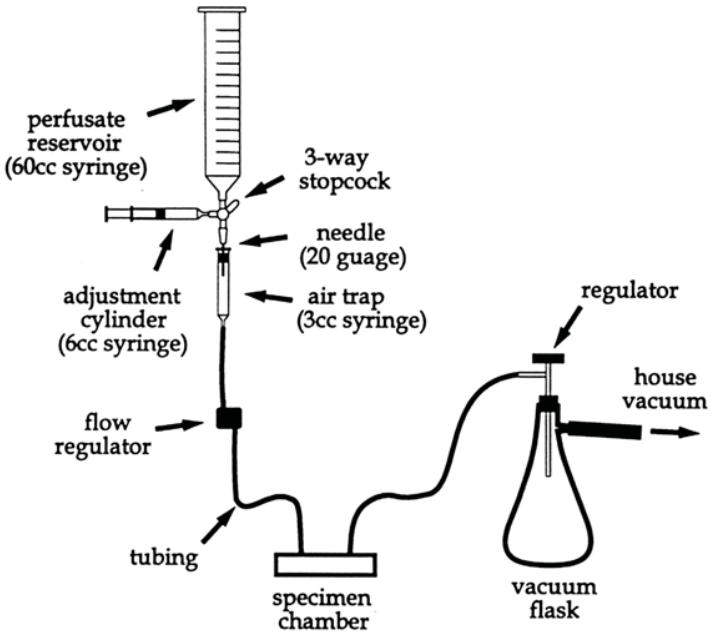

Fig. 19.3 – Schematic diagram of a simple perfusion system for maintaining tissue slices on the microscope stage. The gravity perfusion system is constructed using readily available syringes, three-way stopcocks, and tubing. The perfusion rate is adjusted using a simple thumb-wheel regulator. Perfusate is removed through a vacuum line and flask.

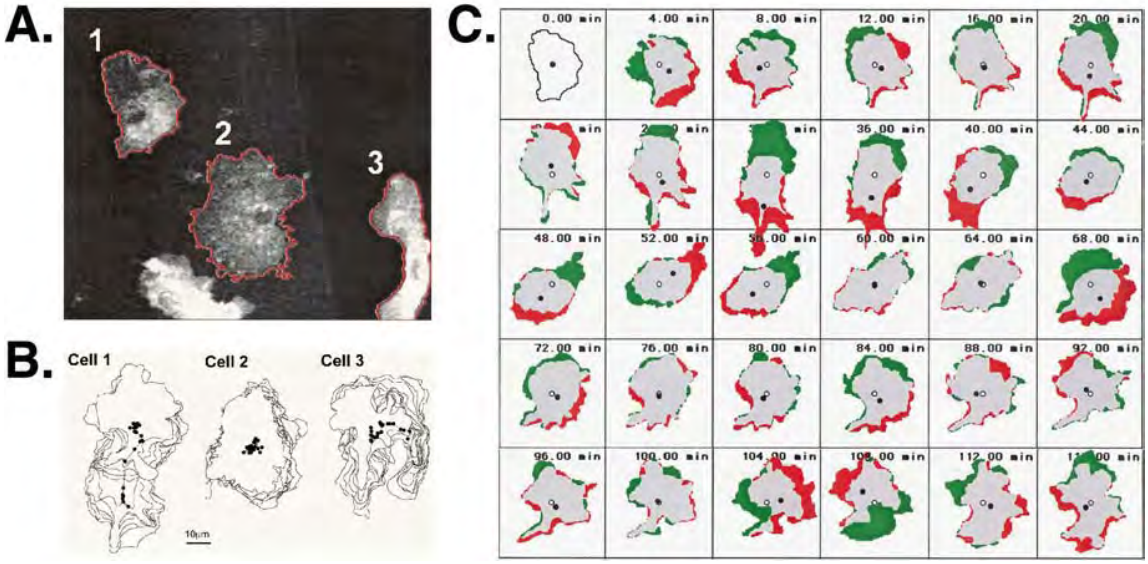

Fig. 19.4 – Use of a Dynamic Image Analysis System (DIAS; Soll, 1995, 1999) to characterize microglial motility behaviors in time-lapse imaging experiments. (A) Boundaries (red lines) of three FITC-IB_4-labeled microglial cells were defined by automated, computer-assisted edge detection. (B) Tracings of the 3 cells show motility behaviors over a 2-hr period. The cell centroid (black dots) were computed and plotted for each time-point. Note that all 3 cells are motile, but cell 2 does not locomote. (C) Areas of new protrusion (green) and resorption (red) are shown for cell 1 at 4 min intervals. The new centroid (open dot) is shown in relation to the former centroid (filled dot).

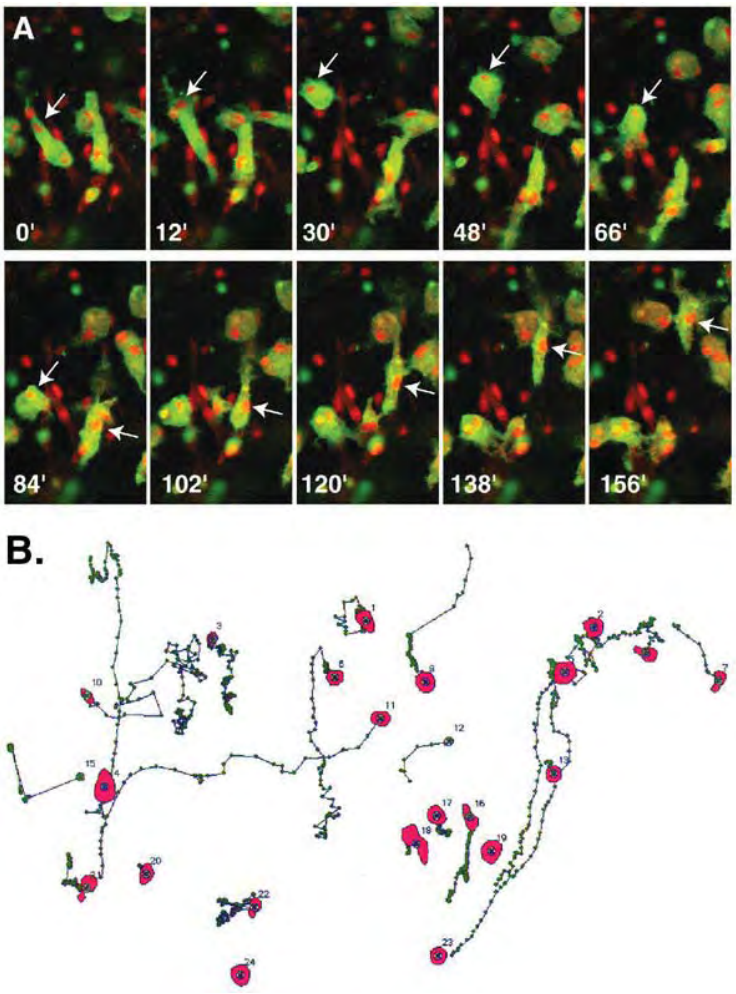

Fig. 19.5 – Two-channel time-lapse imaging of microglia (green) and cell nuclei (red). (A) Time lapse sequence shows capability of simultaneously imaging IB4-labeled microglia and nuclei of cells using a cell membrane permeant DNA binding dye (Syto61). Note the nuclei (arrows) in two different migrating microglial cells. The sequence spans 156 min. Only a small portion of the original field of view is shown. (B) Automated tracing of movement of cell nuclei shows paths taken by nuclei of cells in the experiment show in 'A'. Nuclei were detected by DIAS software (Soll, 1995). Only a select subset of nuclei are shown. Note differences in the movement among different cells.

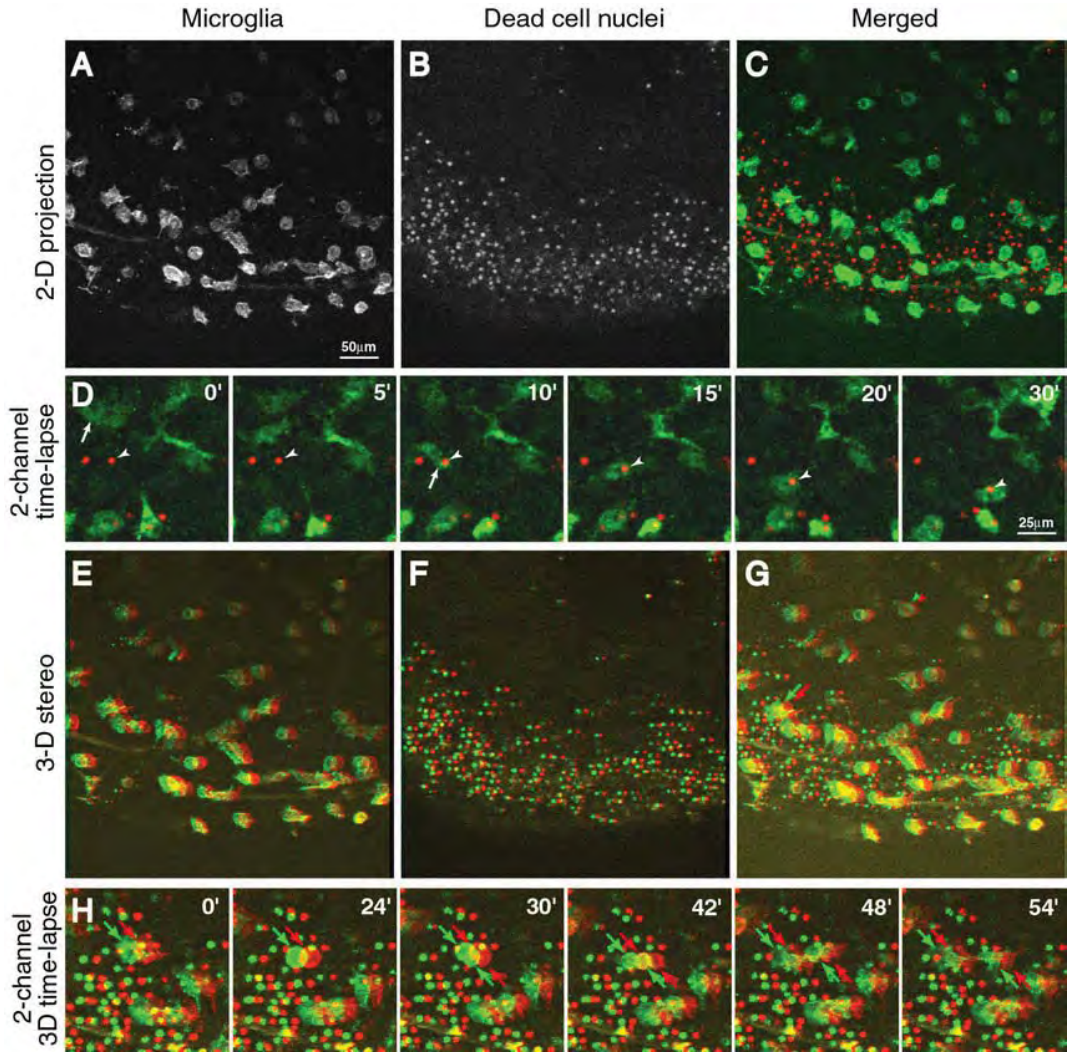

Fig. 19.6 – Two- and three-dimensional, two-channel time-lapse imaging of microglial movements and phagocytosis of dead cell nuclei in live rat brain tissue slice cultures (P6+2DIV). (A) Microglia are stained with a fluorescent lectin, FITC-IB4. (B) Dead cell nuclei are stained with To-Pro-3. (C) Merged image of 'A' and 'B.' The image represents a projection of nine optical sections spanning 40 μm in z-depth. Images were captured using a 20x/0.7 dry objective. (D) Time-lapse sequence of a portion of the field of view above showing phagocytic clearance of a dead cell nucleus (arrowhead) by a locomotory microglial cell (arrow). Note that the microglial cell maintains a rapid rate of locomotion as it sweeps over and picks up the dead cell nucleus. (E-G) 3D stereo projections of images in A-C above. Use red-green glasses (red over left eye) to view depth. (H) 3D stereo time-lapse sequence showing mitosis of a microglial cell (arrow) near the surface of the brain slice culture. The small, round objects represent the condensed nuclei of dead cells. Time is shown in minutes. See the supplemental video movies at http\\. Adapted from Petersen & Dailey, 2004.

TABLE 1: OVERVIEW OF LIVE CELL FLUORESCENCE CONFOCAL IMAGING TECHNIQUES

Technique	Description	Review Article(s)	Selected Examples/References
1. Time-lapse fluorescence imaging.	Repeated imaging of a field of view (single optical section) in live specimen over time.	• Cooper et al., 1999.	• Imaged dynamic changes in fluorescently labeled Golgi membranes (Cooper et al., 1990)
2. Multi-channel or ratiometric time-lapse fluorescence imaging.	Simultaneous or sequential imaging in two or more fluorescent channels over time.	• Stricker, 2004. • Ellenberg et al., 1999.	• Monitored sorting of CFP- and YFP-tagged proteins through the Golgi Apparatus (Keller et al., 2001).
3. Three-dimensional time-lapse (4D) imaging.	Repeated collection of z-series stacks of images over time.	• Gerlich & Ellenberg, 2003. • Bement et al., 2003. • Hammond & Glick, 2000. • Thomas & White, 1998.	• Imaged neuronal dendritic spines in brain slice cultures (Marrs et al., 2001). • Imaged mitosis and migration of developing cortical neurons (Noctor et al., 2004).
4. Three-dimensional multi-channel (5D) time-lapse fluorescence imaging.	Repeated collection of z-stacks in two or more fluorescent channels over time.	• Andrews et al., 2002. • Gerlich et al., 2001.	• Imaged T-cell–dendritic cell interactions in lymph nodes (Stoll et al., 2002). • Microglial phagocytosis in brain slices (Petersen & Dailey, 2004).
5. Spectral imaging and linear unmixing.	Method for discriminating distinct fluorophores with strongly overlapping emission spectra.	• Berg, 2004. • Zimmermann et al., 2003. • Seyfried et al., 2003. • Hiraoka et al., 2002. • Dickenson et al., 2001.	• Unmix spectrally similar fluorophores in plant cells (Berg, 2004) • Resolve multiple fluorescent proteins in vertebrate cells by multiphoton imaging spectroscopy (Lansford et al., 2001)
6. Fluorescence recovery after photobleaching (FRAP).	Measures recovery of fluorescence after bleaching of a portion of the specimen. Recovery may be due to protein diffusion, binding/dissociation or transport processes.	• Lippincott-Schwarz et al., 2003. • Meyvis et al., 1999.	• Used FRAP to study integrin turnover at focal adhesions (Ballestrem et al., 2001)
7. Fluorescence loss in photobleaching (FLIP).	Repeated photobleaching used to determine continuity of cell compartments and mobility of fluorescent proteins within these compartments.	• Lippincott-Schwartz et al., 2001	• Dynamics and retention of correctly folded and misfolded proteins were compared in native ER membranes (Nehls *et al.*, 2000)

8. Fluorescence localization after photobleaching (FLAP).	Method for localized photo-labeling and subsequent tracking of specific molecules bearing two different fluorophores within living cells.	• Dunn et al., 2002.	• Used FLAP to show that actin is rapidly delivered to the leading edge of protruding cells (Zicha et al., 2003).
9. Fluorescence resonance energy transfer (FRET).	Non-radiative energy transfer from a donor to an acceptor fluorophore with overlapping emission and excitation spectra. Useful for measuring interactions between two fluorescently tagged proteins.	• Sekar and Periasamy, 2003 • Wouters et al., 2001	• FRET used to study activation of small G proteins during phagocytosis (Hoppe and Swanson, 2004) • FRET analysis shows that GTP-Rac coupling to effectors is locally enhanced in lamellipodia (Pozo et al., 2002)
10. Fluorescence lifetime imaging (FLIM).	Method to investigate molecular interactions, metabolic reactions, and energy transfer in cells and subcellular structures.	• Peter and Ameer-Beg, 2004. • Periasamy et al., 2002. • Bastiaens and Squire, 1999. • Pepperkok et al., 1999.	• Used FLIM to study interaction between CD44 and ezrin (Legg et al., 2002) • Quantified dimerization of transcription factor CAATT/enhancer binding protein alpha in living pituitary cells (Elangovan et al., 2002)
11. Fluorescence correlation spectroscopy (FCS).	Measures spontaneous fluorescence intensity fluctuations in a microscopic detection volume. Provides information on equilibrium concentrations, reaction kinetics, and diffusion rates of molecules.	• Bacia & Schwille, 2003. • Hess et al., 2002. • Elson, 2001.	• Compared mobility and molecular interactions between CaM and CaMKII in solution and in living cells (Kim et al., 2004)
12. Fluorescence speckle microscopy.	Uses very low concentration of fluorescent subunits to reduce out-of-focus fluorescence and improve visibility of fluorescently labeled structures and their dynamics in thick regions of living cells.	• Adams et al., 2003 • Waterman-Storer et al., 1998	• Studied coupling of microtubule and actin movements in migrating cells (Salmon et al., 2002)
13. Photo-uncaging/ Photoactivation.	Photo-induced activation of an inert molecule to an active state (e.g., release of a caging group from a 'caged' compound), or activation of a photoactivatable fluorescent protein (e.g., PA-GFP, Kaede).	• Patterson & Lippincott-Schwartz, 2004 • Park et al., 2002. • Dorman and Prestwich, 2000. • Politz, 1999.	• Photo-release of caged Ca^{2+} in brain astrocytes regulates vascular constriction (Mulligan and MacVicar, 2004) • Used a reversible photoactivatable fluorescent protein to study nuclear import and export of ERK1 and importin (Ando et al., 2004)

14. Optical tweezers/laser trapping.	Uses the 'radiation pressure' of a stream of photons emitted from an infrared laser to "trap" small objects and molecules.	• Kuo, 2001. • Schwarzbauer, 1997.	• Studied strength of cadherin adhesions in endothelial cells (Baumgartner et al., 2003)
15. Fast physiological imaging. • Full field • Line-scanning	Rapid, repeated collection of single scan lines or 2D images of specimen labeled with physiological indicators.	• Rubart, 2004. • Wang et al., 2004. • Ashworth, 2004. • Niggli & Egger, 2004. • Miyawaki, 2003.	• Imaged Ca^{2+} sparks in muscle fibers (Hollingworth et al., 2000; Brum et al., 2000). • Ca^{2+} imaging in neuronal dendritic spines (Pologruto et al., 2004).
16. Combined fluorescence and transmitted light imaging.	Repeated simultaneous collection of one or more fluorescent channels and a transmitted light channel (e.g., DIC).	• Cogswell & Sheppard, 1991, 1992.	• Imaged chromatin dynamics during the formation of the interphase nucleus (Manders et al., 2003) • Imaged E-cadherin-GFP accumulation at cell adhesions in epithelial cells (Adams et al., 1998).

TABLE 19.2: EXPERIMENTAL CONSIDERATIONS FOR LIVE CELL IMAGING

Consideration	Problem	Potential solution(s)
1. Temperature	Many biological phenomena are temperature sensitive.	Use stage heaters; inline perfusion heaters; objective lens heaters; environmental boxes.
2. Oxygenation	Most live biological specimens require O2 (and removal of CO2) to remain healthy. Oxygen may become depleted in closed chambers.	Use a perfusion chamber. Exchange used chamber media with oxygenated media intermittently or continuously. Increase volume of chamber to promote health.
3. pH	Metabolism of live biological tissues can induce severe pH changes in chamber media over time.	• Monitor chamber pH. • Use HEPES (10-25mM)-buffered media. • Exchange chamber media intermittently or continuously (perfusion). • Use media without phenol red pH indicator.
4. Humidity	Stage heating (especially with forced air) may cause evaporation from an open chamber, leading to dramatic changes in salinity and pH.	• Use closed chamber configuration (perfusion chamber). • Use humidified environmental box.
5. Fluorescence signal strength	Weakly fluorescent probes or low concentration of probes can yield weak signals that produce images with low signal-to-noise ratio.	• Increase pixel dwell time. • Open confocal pinhole aperture (e.g., to >2 Airy disks). • Maximize throughput of emission pathway (e.g., in spectral imaging systems with variable spectral filters) • Use line or frame averaging to improve signal-to-noise ratio. • Adjust illumination (filling) of back aperture of objective lens.
6. Channel bleed-through or cross-talk	In biological specimens labeled with multiple fluorescent probes, signals from one channel may be detected in other channels.	• Image separate fluorescence channels sequentially (either line-by-line or frame-by-frame in scanning systems). • Use spectral imaging and linear unmixing algorithms.
7. Photobleaching	Fluorescent probes bleach with repeated illumination. Some fluorescent probes bleach quickly.	• Reduce incident illumination. • Open confocal pinhole aperture. • Maximize throughput of emission pathway (e.g., in spectral imaging systems with variable spectral filters). • Reduce pixel dwell time (in scanning systems). • Reduce frequency of image capture. • Blank laser beam during flyback (in scanning systems).
8. Spatial Resolution	Some observations require very high spatial resolution in x-y or z.	• Use high NA objectives. • Reduce size of confocal pinhole aperture (to ~1 Airy disk). • Increase spatial sampling frequency (guided by Nyquist

		theorem). • Increase electronic zoom (but avoid empty magnification). • Decrease step size in z-stacks. • Use water immersion objective lenses in thick specimen to reduce spherical aberrations. • Deconvolve the images.
9. Temporal resolution	Some biological phenomena are rapid relative to the rate of image collection (especially problematic with laser scanning confocal systems).	• Reduce field of view (e.g., collect fewer horizontal lines) • Reduce pixel dwell time (e.g., increase scan speed). • Reduce spatial sampling frequency (e.g., reduce pixel array from 1024 to 512).
10. Focus drift	Live biological specimen on heated microscope stages, or features within live specimens (e.g., mitotic cells), can move relative to a fixed focal plane.	• Collect a z-stack of images, and reconstruct these images following the observation. • Manual focus adjustments may be required periodically. • Auto-focus methodology may be employed in some cases.

TABLE 19.3: COMMERCIALLY AVAILABLE CHAMBERS FOR LIVE CELL IMAGING

Source	Description/Features	Contact Info
20/20 Technology, Inc. Bldg. 2, Unit A 311 Judges Road Wilmington, NC 28405 USA	Heating, cooling, atmosphere control instrumentation for microscopy.	TEL: 1-910-791-9226 WEB: http://20-20tech.com/ EMAIL:
ALA Scientific Instruments Inc. 1100 Shames Dr. Westbury, NY 11590 USA	Microincubators and temperature control; Peltier heating & cooling pre-stage; recording chambers; inline perfusion heating tube.	TEL: 516-997-5780 WEB: www.alascience.com EMAIL: sales@alascience.com
ASI / Applied Scientific Instrumentation Inc. 29391 W. Enid Rd. Eugene, OR 97402 USA	Supplier for Solent and Bioptechs incubation chambers.	TEL: 541-461-8181 WEB: http://www.asiimaging.com/ EMAIL: info@ASIimaging.com
AutoMate Scientific, Inc. 336 Baden Street San Francisco, California 94131 USA	Programmable controlled perfusion systems, temperature control, valves and fittings, oocyte perfusion chamber, Petri dish perfusion chamber, sub-millisecond switching, submerged and interface tissue and brain slice chambers.	TEL: 415-239-6080 WEB: http://www.autom8.com/ EMAIL: info@autom8.com
Bellco Glass, Inc. 340 Edrudo Road, Vineland, NJ 08360 USA	Sykes-Moore culture chambers; used with stationary culture when medium is changed intermittently.	TEL: 1-800-257-7043 WEB: http://www.bellcoglass.com/ EMAIL: cservice@bellcoglass.com
BioCrystal Ltd. OptiCell 575 McCorkle Blvd. Westerville, OH 43082 USA	OptiCell™ is a sterile, sealed cell culture environment between two optically clear gas-permeable growth surfaces in a standard microtiter plate-sized plastic frame with ports for access to the contents.	TEL: 614-818-0019 WEB: www.opticell.com EMAIL: sales@opticell.com
Bioptechs, Inc. 3560 Beck Road Butler, PA 16002 USA	Live cell microscopy environmental control systems. Thermal regulation of specimen and objective, electronic control and integration of temperature and perfusion.	TEL: 724-282-7145 WEB: http://www.bioptechs.com/ EMAIL: info@bioptechs.com
Bioscience Tools – CB Consulting Inc., 4527 52nd Street, San Diego, CA 92115 USA	Glass bottom Petri dishes; ultra-thin imaging chambers; temperature control; perfusion systems; small volume delivery systems; ultra-fast temperature/solution	TEL: 1-877-853-9755 WEB: http://biosciencetools.com/ EMAIL: info@biosciencetools.com

	switch.	
C&L Instruments, Inc. 314 Scout Lane Hummelstown, PA 17036 USA	Fluorometers and fluorometer components for steady-state fluorescence measurements; complete fluorescence systems for photometry and fluorescence imaging.	TEL: 1-717-564-9491 WEB: http://www.fluorescence.com/ EMAIL: support@fluorescence.com
CellBiology Trading Hinsbeker Berg 28a Hamburg, 22399 Germany	Microinjection and incubation; EMBL live cell observation chamber.	TEL: 49-0-40-53889432 WEB: http://cellbiology-trading.com/ EMAIL: info@cellbiology-trading.com
Dagan Corporation 2855 Park Avenue, Minneapolis, Minnesota 55407 USA	Microscope stage temperature controller; perfusion controller.	TEL: 612-827-5959 WEB: http://www.dagan.com/ EMAIL: support@dagan.com
Digitimer Ltd 37 Hydeway Welwyn Garden City Hertfordshire, AL7 3BE, England	AutoMate Scientific, Medical Systems and Scientific Systems Design incubators, chambers, and perfusion systems.	TEL: +44 (0) 1707 328347 WEB: http://www.digitimer.com/ EMAIL: sales@digitimer.com
Grace Bio-Labs, Inc. P.O. Box 228 Bend, OR 97709 USA	Manufactures 3-D microporous coatings on microscope slides, and a variety of "press to seal" enclosures for microarrays, cell culture, and high throughput cytochemistry, hybridization, cytogenetics, and fluorescent imaging applications.	TEL: 1-800-813-7339 WEB: http://www.gracebio.com/ EMAIL: custservice@gracebio.com
Harvard Apparatus Inc. 84 October Hill Rd. Holliston, MA 01746 USA	Variety of valve controlled perfusion systems.	TEL: 508-893-8999 WEB: http://www.harvardapparatus.com EMAIL: bioscience@harvardapparatus.com
Integrated BioDiagnostics Schellingstrasse 4 80799 München, Germany	The µ-slide family of live cell imaging flow chambers; suited for optical studies of hydrodynamic shear stress on biofilms or adhesion studies on cell layers.	TEL: +49 (0)89 / 2180 64 19 WEB: http://www.ibidi.de/ EMAIL: info@ibidi.de
Intracel, Ltd. Unit 4 Station Road Shepreth, Royston Herts, SG8 6PZ England	WillCo glass bottomed dishes; Bioptechs micro-environmental control systems.	TEL: 01763 262680 WEB: http://www.intracel.co.uk/ EMAIL: intracel@intracel.co.uk
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OPINION

Has the revolution arrived?

Looking back over the past decade of human genomics, **Francis Collins** finds five key lessons for the future of personalized medicine — for technology, policy, partnerships and pharmacogenomics.

On 26 June 2000, Craig Venter and I stood next to the President of the United States, in the same room of the White House where the explorers Meriwether Lewis and William Clark had unfurled their map of the Northwest Territories for Thomas Jefferson. "Today," Bill Clinton said, "the world is joining us here in the East Room to behold a map of even greater significance. We are here to celebrate the completion of the first survey of the entire human genome … With this profound new knowledge, humankind is on the verge of gaining immense, new power to heal. Genome science will have a real impact on all our lives — and even more, on the lives of our children. It will revolutionize the diagnosis, prevention, and treatment of most, if not all, human diseases."

I was honoured to be standing there, but also somewhat embarrassed: the milestone being reported was not yet attached to a publication — there was a lot of analysis still to do, and the paper would not appear in *Nature* until eight months later. Still, it was a heady moment.

Wisely, the president did not attach timetables to his bold predictions, even though in the early days of the millennium, everyone wanted to hear where this genome revolution was going. I even made my own predictions for 2010. Never having discarded a PowerPoint file, I can reproduce my list verbatim:

- Predictive genetic tests will be available for a dozen conditions
- Interventions to reduce risk will be available for several of these
- Many primary-care providers will begin to practise genetic medicine
- Preimplantation genetic diagnosis will be widely available, and its limits will be fiercely debated
- A ban on genetic discrimination will be in place in the United States
- Access to genetic medicine will remain inequitable, especially in the developing world

It is fair to say that all of these predictions have come true, with some caveats that offer important lessons about the best path forward for genomics and personalized medicine. The promise of a revolution in human health remains quite real. Those who somehow expected dramatic results overnight may be disappointed, but should remember that genomics obeys the First Law of Technology: we invariably overestimate the short-term impacts of new technologies and underestimate their longer-term effects.

Breathtaking acceleration

The decade from 2000 to 2010 was characterized by breathtaking acceleration in genome science. Thanks to advances in DNA sequencing technology that dropped the cost approximately 14,000-fold between 1999 and 2009, finished sequences are now available for 14 mammals, and draft or complete sequences have been done for many other vertebrates, invertebrates, fungi, plants and microorganisms. Comparative genomics has emerged as a powerful approach for understanding evolution and genome function at a level of detail barely imagined a few years ago.

For humans, the HapMap project produced a remarkable catalogue of common variation in the genome in just three years, from 2002 to 2005. As full sequencing has become more practical, researchers have been releasing complete genomes of individuals — a total of 13 at the time of this writing, including my personal hero, Archbishop Desmond Tutu of South Africa. In 2011, an international team is set to complete the data-production phase of the 1000 Genomes Project, designed to produce highly accurate assembled sequences from more than 1,000 individuals whose ancestors came from Europe, Asia and Africa.

> "This profusion of therapeutic opportunites is a challenge to prioritize."

The same determination to study the entire genome, not just isolated segments, has now been applied to understanding its function — although this quest is, of course, much more complicated and open-ended. The Encyclopedia of DNA Elements (ENCODE) project (started in pilot form in 2003 and slated to run at least until 2011) and the US National Institutes of Health (NIH) Roadmap Epigenomics Program (started in 2008 and funded until 2013) continue to define the 'parts list' of the human genome. These projects identify the locations of genes (protein coding and non-coding) and the patterns that determine whether genes are switched on or off in a given tissue — patterns of chromatin modification, transcription factors and DNA methylation.

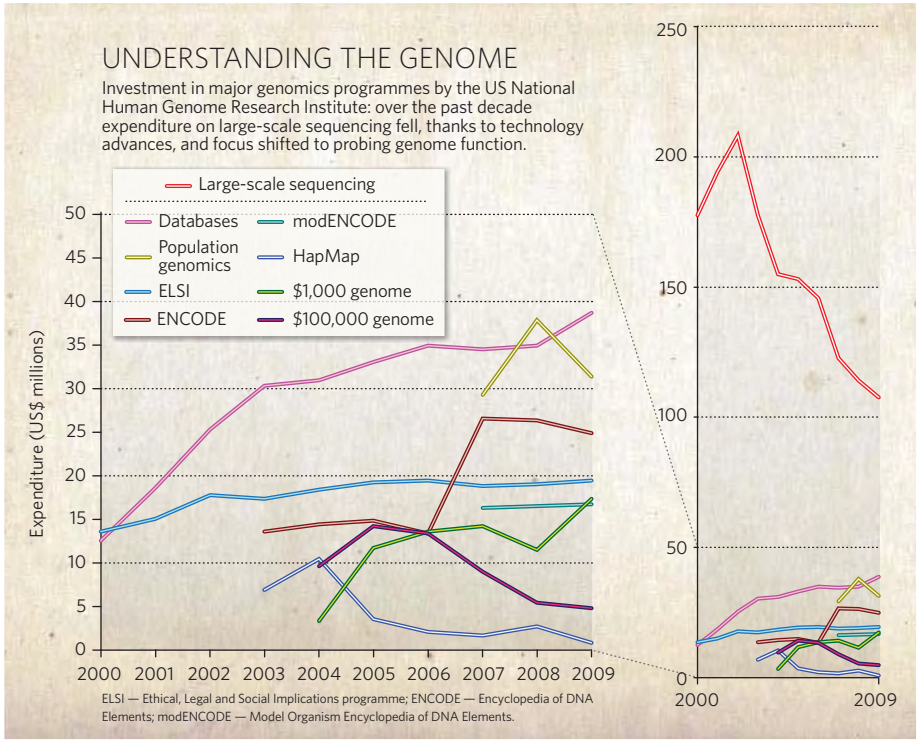
With regard to medical applications, genome-wide association studies (GWAS) have now revealed an astounding number of common DNA variations that play a part in the risk of developing common diseases such as heart disease, diabetes, cancer or autoimmunity. To identify less common variations, methods to target DNA sequencing to subsets of the human genome have been developed. These methods can now sequence 80–90% of the protein-coding regions — the exons or 'exome' — of a human DNA sample for just a few thousand dollars.

Genome research has already had a profound impact on scientific progress. The combination of new technologies and freely accessible databases of high-quality genomic information has enabled the average investigator to make discoveries much more quickly than would otherwise have been possible. For example, the search for the cystic fibrosis gene finally succeeded in 1989 after years of effort by my lab and several others, at an estimated cost of US$50 million. Such a project could now be accomplished in a few days by a good graduate student with access to the Internet, appropriate DNA samples, some inexpensive reagents, a thermal cycler and a DNA sequencer (see graphic).

The consequences for clinical medicine, however, have thus far been modest. Some major advances have indeed been made: powerful new drugs have been developed for some cancers; genetic tests can predict whether people with breast cancer need chemotherapy; the major risk factors for macular degeneration have been identified; and drug response can be predicted accurately for more than a dozen drugs. But it is fair to say that the Human Genome Project has not yet directly affected the health care of most individuals.

GWAS have so far identified only a small fraction of the heritability of common diseases, so the ability to make meaningful predictions is still quite limited, even using chips that test for a million or more common variants. Nonetheless, direct-to-consumer marketing of genetic risk prediction, based on the rapidly growing

M. GUYER, NHGRI



database of GWAS results, is attracting early adopters. Having gone through that process myself, I can report that I found the opportunity to view my own personal genotype results rather riveting, despite the limited clinical validity and utility of many of these predictions.

This dynamic is likely to change in the next five years. Much of the missing heritability (the 'dark matter' of the genome) will probably turn up as the technology advances. Whole-genome sequencing, coming into its own as the cost per genome falls below $1,000 in the next three to five years, will identify rare variants of larger effect and the copy number variants that GWAS may have missed. With an increasing inventory of these discoveries, prediction of disease risk and drug response will continue to improve.

As the cost falls and evidence grows, there will be increasing merit in obtaining complete-genome sequences for each of us, and storing that information, with appropriate privacy protections, in our medical records, where it will be quickly available to guide prevention strategies or medication choice.

Perhaps the most profound consequence of the genome revolution in the long run will be the development of targeted therapeutics based on a detailed molecular understanding of pathogenesis. However, this is also the goal most challenged by long timelines, high failure rates and exorbitant costs. Despite those obstacles, inspiring examples of success are in hand, many of them (trastuzumab, imatinib, gefitinib and erlotinib) for the treatment of cancer. Furthermore, the identification of new cancer drug targets is accelerating rapidly as a consequence of the ability to do deep genome sequencing of many tumours to identify recurrent mutations. Projects such as the Cancer Genome Atlas, which is carrying out the equivalent of 20,000 genome projects on matched tumour and blood DNA samples from 20 common types of cancer, have begun to reveal numerous opportunities for therapeutic development. And GWAS have pointed to hundreds of previously unrecognized drug targets for dozens of other diseases.

This profusion of therapeutic opportunities is a challenge to prioritize. Efforts are now under way to forge innovative partnerships between the traditional strengths of the private sector and academic labs. The NIH has provided new resources to catalyse such partnerships, including access by academic investigators to high-throughput screening through the Molecular Libraries Roadmap project, and to preclinical testing of promising lead compounds through the Therapeutics for Rare and Neglected Disease initiative.

Enabling the future

I propose five major lessons that could be gleaned from this first decade of the genome era. First, free and open access to genome data has had a profoundly positive effect on progress. The radical ethic of immediate data deposit, adopted by the Human Genome Project in 1996 and now the norm for other community resource projects, empowers the best brains on the planet to begin work immediately in analysing the massive amounts of genomic data now being produced. It is a very good thing that the 'race for the genome' in 1998–2000 resulted in the human genome sequence being immediately and freely available to all, rather than becoming a commercial commodity.

Second, technology development for sequencing and functional genomics — key to the success achieved thus far — must continue to be a major focus of investment by both the public and private sectors. Although huge leaps have been made in increasing the speed and reducing the costs of DNA sequencing, expression analysis and methods to assess the epigenome, the limits are still nowhere near being reached.

Third, the success of personalized medicine will depend on continued accurate identification of genetic and environmental risk factors, and the ability to utilize this information in the real world to influence health behaviours and achieve better outcomes. This will require well designed, large-scale research projects, for discovering risk factors and for testing the implementation of prevention and pharmacogenomic programmes.

Fourth, achieving the enormous promise of the myriad new drug targets emerging from genomic analysis of common and rare diseases requires new paradigms of public–private partnership. Academic investigators will have a much more important role in the early stages, effectively 'de-risking' projects for downstream commercial investment. Closer relationships between the US Food and Drug Administration and the NIH, announced this February, will assist this process.

Finally, good policy decisions will be crucial to reaping the benefits that should flow from the coming revelations about the genome. These will include protection of individual privacy, effective education of health-care providers and the public about genomic medicine, and appropriate health-care system reimbursement for the cost of validated preventive measures.

In *The Wisdom of the Sands*, author Antoine de Saint-Exupéry wrote: "As for the future, your task is not to foresee, but to enable it." Genomics has had an exceptionally powerful enabling role in biomedical advances over the past decade. Only time will tell how deep and how far that power will take us. I am willing to bet that the best is yet to come. ■

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See Editorial, page 649, and human genome special at www.nature.com/humangenome.

Systems Biology's Awkward Adolescence

By John Russell

Sept. 13, 2007 | For a decade or more, systems biology has struggled to establish itself as something important. Nudged into existence by powerful new instruments spewing out data and advancing computational power, systems biology (SB) is entering an awkward adolescence during which much of its value will be revealed, but remain far from fully utilized.

The label "systems biology" is pretty awful, except, of course, for the many even worse labels that have been tried. More important is what SB seeks to do: transform biology and health care into a rigorous, predictive science offering a richer understanding of biology and a vastly improved approach to drug development and medicine. SB would build on the molecular biology revolution and elucidate the wiring diagrams (and their rules) buried in the data.

Perhaps the most ambitious framing of the potential of SB is the "P4 Medicine" vision of Institute for System biology founder Lee Hood (see "ISB Is a Sure Cure," *Bio•IT World*, August 2007, p. 42) - predictive, personalized, preventive, and participatory. More prosaically, SB eschews the narrow focus on individual ligand-target interactions, and emphasizes specifying entire biological networks in mechanistic detail. In theory, researchers could identify the best intervention points, the optimum agent(s), definitive biomarkers, and sidestep toxic side effects. It should also be possible to simulate network models to conduct "virtual" drug discovery and clinical trials. (See "What Is Systems Biology?")

While big pharma wrestles with the relevance of SB and how to adopt it organizationally, a few true-believer start-ups are exploiting SB and at least one — Merrimack Pharmaceuticals — has compounds in the clinic. Circling the drug-makers is a diverse gaggle of SB technology providers, many struggling to scale up their fee-for-service and software licensing business models and wondering if they must become drug-makers to flourish long-term. Meanwhile, inside academia and government labs, SB is suddenly gaining ground (See "Systems Biology Is Hot...").

Mixed Signals

Big pharma may pay the lion's share of SB bills, but lately it's sending mixed signals. Early SB advocates Novartis and GlaxoSmithKline have disbanded or deemphasized internal systems biology 'departments.' SB didn't produce the desired bang-for-the-buck, while harsh financial realities have left little wiggle room for maintaining long-term technology bets.

"Systems biology at NIBR [Novartis Institutes of BioMedical Research] is no longer thought of as something distinct from the other disciplines which are brought to bear on our drug discovery process, such as biology, chemistry, and pharmacology. It is tightly integrated into all stages of the drug discovery process," Liam O'Connor, director of quantitative biology at NIBR, told *Bio•IT World* in a wan e-mail.

Other observers suggest Novartis — like many of its Big Pharma brethren — is simply externalizing the risk through collaboration, which is not unreasonable. Even Hood praises Novartis as forward thinking and thinks it would make a good partner.

By contrast, Pfizer, with its $7.4 billion R&D budget, is actually expanding its internal systems biology program as part of a larger company-wide reorganization set in motion by CEO Jeffrey Kindler in January, which emphasizes therapeutic area centers at the expense of global technology centers.

Pfizer might change its SB tune if costs mount and results do not, but David de Graaf, director of systems biology at Pfizer, is betting SB's future inside Pfizer is robust. The SB organization now "runs all the way into the early phases in the clinic rather than just providing candidate drugs," he says. "It now needs to go through essentially proof of principle." Pfizer management is setting up a centralized group to develop SB approaches at the RTC (see "Setting the Systems Bio Syllabus," *Bio•IT World*, Jan. 2007, p. 18). "The site mission is now being focused on deep knowledge of pathways, targets, and compounds," says de Graaf.

The Pfizer group is two years old, and will expand from eight to around thirty researchers. The wealth of local talent has made recruiting the necessary multi-discipline expertise easier, de Graaf says. Pfizer recently recruited former NIBR computational biologist, Carolyn Cho.

Early success in biologics projects and toxicity prediction, says de Graaf, has helped drive SB's acceptance at Pfizer. "This move is a chance for us to show more broadly what systems biology can do for Pfizer," says de Graaf. "We are beyond the tantalizing hints stage. The work we've done on hepatic injury, which we hope to publish very soon, helped demonstrate that. We don't only want to show that we can sustain that and grow that incrementally. We want to go into some new areas and really change people's perceptions. Ultimately we hope to start to grow small groups doing systems biology at all the sites."

Pfizer's SB team will focus on biotherapeutics and predictive tox. De Graaf says that pharma's classical model is to invest heavily in a pathway "and have 8 or 10 projects that are all essentially directed against the same biological mechanism. We hope to not only [reduce the project] number but also to pick the ones that will be winners. That will eventually limit [SB's] application to particular therapeutic areas. Logical bets would be places like cancer and diabetes and maybe inflammation."

Lilly is also committed to retaining a robust internal SB effort, and recently announced a major expansion of its SB activities in Singapore. William Chin, Lilly's VP of discovery, says the company studies protein interactions in rodent models before evaluating their usefulness in humans. "If we could just bridge that gulf a little bit better, if we could be more predictive, we'd be much better off," says Chin. "Looking at pathways and systems is a better way of doing it. The whole idea is that these systems and pathways are probably better conserved than any single molecular target."

Lilly's Singapore center has produced several proprietary markers, says Chin, using some cancer pharmacokinetics and pharmacodynamic modeling. This has helped Lilly convince the FDA that, "as we enter the clinic that we have chosen the right doses."

Merck also has early SB successes. In an upbeat review in the *Journal of Lipid Research* last year, Rosetta (Merck subsidiary) researchers Eric Schadt and Pek Lum suggested that reconstructed networks, "provide a richer context in which to interpret associations between genes and disease. Therefore, these networks can lead to defining pathways underlying disease more objectively and to identifying biomarkers and more robust points for therapeutic intervention."

Colin Hill, CEO and co-founder of Gene Network Sciences, says, "They are bringing the power of SB and genomics to bear on major diseases such as metabolic syndrome with rather striking results. [Schadt and Lum] essentially claimed to have solved metabolic syndrome with SB and genomic techniques. This paper suggests a big win for Merck after five years of work."

Here Comes the Flood

So why aren't the floodgates opening?

One persistent challenge is organizing internal SB efforts to ensure the work is done effectively and is deemed important enough to change attitudes. Should SB groups be centralized, or is it better to build SB skill sets inside therapeutic groups? If centralized, should it act as service centers or do independent work?

"I don't think anyone has the recipe for how to do it," says Ruedi Aebersold, co-founder of ISB and currently the leader of Systems X.ch in Switzerland (See "Systems Biology Is Hot..."). A key asset at the ISB, Aebersold recalls, was that "people are close by and communicate, and not just in a WebEx meeting." Communication thrived across departments, groups, and disease areas and, this being Seattle, "they actually run into each other, they sit together at the coffee table and interact informally.... That was an extremely striking experience at ISB that I've never experienced before or since," says Aebersold.

But Jack Beausman, principal scientist, pathway capabilities at AstraZeneca, feels that SB "will be done within specialized groups." Trying to transfer that thinking to super-users and biologists, he says, hasn't been terribly successful. "At this point it's an art that's only going to be successful for the next 4 or 5 years within specialized groups, specialized companies for that matter," he says.

Beausman points out that big pharma's investment in SB is miniscule compared to what it's spending on companies with promising compounds. His own company spent $14 billion this year to acquire MedImmune. "I don't think people view in the very near future that systems biology, and certainly modeling, have any transformative potential. It's incremental," says Beausman.

Not that incremental progress is bad. Modeling is almost always involved in the target decision-making process at AZ, reports Beausman, and his small group will grow slightly this year. "We're not really identifying targets. That's very hard," he admits. Rather, his group looks at a particular system, such as angiogenesis, and asks: "What would be good points at which to intervene?" Adds Beausman: "Sometimes you can identify points of attack that are clearly not going to work in systems like that, and that's valuable information. Sometimes we don't know enough and can't say anything."

Prying SB success stories from close-mouthed pharma is a challenge. Moreover, most early SB-related successes have been go/no-go decisions. While halting problematic programs early saves (big) money, it doesn't excite drugmakers the way identifying the next blockbuster would.

Alex Bangs, CTO and co-founder of bio-simulation specialist, Entelos, says his firm's impact has "been in cases where we've predicted that a target was not going to be effective," resulting in the project being stopped. Says Bangs: "We've had people say, 'Well gee, we keep giving people negative news about things.' And the answer is, 'Well, a lot of stuff fails.' So it's not surprising that there are a lot of negative answers."

Landing truly important work is another hurdle. Therapeutic groups are often suspicious for many reasons, including reluctance to share credit or control, and internal reward systems that actually financially penalize therapeutic areas for such collaborations. At Pfizer, de Graaf is working with portfolio managers in therapeutic areas to identify projects. These decision makers worry more about advancing important projects than preserving pet projects or turf wars. He stresses a healthy dose of diplomacy is always needed.

In the end, Hood probably has right perspective: "What will convince [pharma] are examples of drugs that came out of these approaches that were produced rapidly and economically. The only question is whether or not, after you get those examples, it's too late for them to get in the ball game... There are going to be a lot of younger companies who see that this is the way to do things."

Hood says pharma has tended to merge with these companies, frequently taking the product and throwing the company away. "Whether they can merge with a company and retain its unique individuality is a question," he says. "I know of only one example (Roche/Genentech) when that's been done and that was done superbly."

The Merrimack Method

Merrimack Pharmaceuticals, formed six years ago by a multidisciplinary group of MIT and Harvard researchers, is using SB to find drugs for cancer and autoimmune disease. The company was spun out of a DARPA program designed to get engineers, mathematicians, and biologists to do biology differently. Co-founder Ulrich Nielsen, VP of research, was a postdoc with Peter Sorger at MIT. "There was a core of people, especially chemical engineers and biologists, who saw new avenues, not just to understand biology but to change the way drug discovery is done."

While most SB spinouts have opted to become tool companies, Merrimack bucked this trend. It also didn't take VC money, yet raised $145 million over five years from other sources (which Nielsen declines to name). Headcount has grown to about 75. Merrimack espouses an approach called 'network biology' to better therapeutics. The company says it has "developed a proprietary technology platform that enables the high throughput profiling of complex biological systems... Network Biology seeks to understand the system dynamics that govern protein networks — the functional set of proteins that regulate cellular decisions. More than a research tool, Network Biology is a set of technologies designed to enhance the entire drug discovery, development, and commercialization process."

Merrimack's models are mechanism-based, initially informed by the literature, but quickly enhanced by the company's extensive (and expensive) wet-lab to generate large data sets. "These are differential equation type models that describe molecular interactions by knowing the concentrations and rate constants for how proteins

interact," says Nielsen. "You can build up fairly large models of the system you're working with, and importantly, you can use those models to simulate problems that are not necessarily in your data set.

"We've spent a lot of our focus on growth factors in cytokine signaling. We can ask questions like, how do you actually best inhibit a given pathway, and look at what are the most sensitive targets in the pathway. There are some really nice computational tools for doing that."

Merrimack is focused exclusively on biologics. Its first candidate, MM-093, which targets rheumatoid arthritis, psoriasis, and multiple sclerosis, is in-licensed from McGill University and is not fully representative of the company's approach. The RA project is in phase II trials. Of three other homegrown cancer candidates, MM-121 should enter phase I this winter. "It was the first pathway we built," says Nielsen, "and despite being a very-well trodden area, we found insights that we believe are important and I think we've gotten a few years leap."

Merrimack has modeled signaling networks regulating the biology of solid tumors, and reports developing several novel approaches to target the signals driving tumor growth. Companion diagnostics also figure prominently in Merrimack's planning, particular given the heterogeneity of cancer. Says Neilsen: "Network biology is giving us a new view on cancer that's not necessarily defined by ...growth physiology or even pathology. Whether the tumor comes from breast or ovarian tissue or colon, doesn't always define the molecular pathology behind it."

Unlike many *in silico*-mostly SB plays, Merrimack depends heavily on its experimental biology and also intends to manufacture its product as well. Nielsen says the company has embraced multiplexing technologies and robotics for high-throughput analysis of protein expression and phosphorylation. "Experimental biology is obviously much more expensive than literature mining and computational biology, so yeah, we invested heavily in method development, equipment, and people," he says.

Nielsen offers an opinion why big pharma has been less successful in adopting SB. "I think most pharmaceutical companies, at least on the surface, are embracing these technologies, often by establishing systems biology institutes or departments." By contrast, Nielsen says Merrimack stresses "the integration of this approach into the whole of drug discovery, and in fact, bringing these tools into simulating problems in drug discovery before you create a drug." Whereas pharma has applied SB in later stages, for example identifying responders in clinical trials, this is "a very different paradigm from sort of engineering things de novo, which is what we're doing," Nielsen says.

Another example is Avalon Pharmaceuticals, which has had a successful IPO. Leveraging gene transcription and proprietary cell-based assay technology, Avalon's lead candidate IMPDH (inosine monophosphate dehydrogenase) inhibitor, AVN944, is in phase I trial for heme malignancies and expected to move into Phase II trials soon.

Clinical trials are far from having a proven drug, and that's the fuel that will truly ignite the systems biology fire. No drugs, no bonfire. But everyone seems to agree the inferno is coming, just not on when.

Growing Pains

Technology advances and cost reduction are still needed. Perhaps more knowledge is needed too, though Nielsen scoffs at this idea. So too does Douglas Lauffenburger, director of MIT's department of biological engineering, and an architect of many of the ideas being pursued by Merrimack. "I am entirely confident about the bright future of systems biology, even if some pharmas bail out in the short run," says Lauffenburger. "This is a long-term revolutionary approach to biology, so it is patently infeasible for it to 'dissipate.'"

Gustavo Stolivitsky, manager of functional genomics and systems biology at IBM's T.J. Watson Research Center, concedes that, "systems biology hasn't delivered some big clear success story.... But I think everybody knows that in the long run, it will be one of the ways to go, if not the only way to go."

Aebersold hesitates to offer long-term projections but then reconsiders: "I don't see one particular inflection. I think there are many inflections whenever someone comes up with a new technology that measures, let's say, some contextual relationships or a software, an algorithm. It's not an endpoint like the genome project, where if you're Bill Clinton you go and stand in front of the camera and say, now it's been done [even if it hasn't], and have a press conference."

In some ways, systems biology is both a philosophy and a methodology. The philosophy is pretty clear; try to understand in fine mechanistic detail how living systems work as integrated entities, from pathways to networks to whole cells and ultimately the entire organism. That doesn't sound so different than just plain biology, though a change in language is sometimes needed to catalyze actions.

The methodology is more about coordinated use of the diverse tools required to connect the dots the philosophy wishes to understand. In practical terms today, it's primarily tools to interrogate molecular (and other biochemical) systems *and* computational power and math to make sense of the data. In this sense, many companies have fastened onto various technologies and championed their use as demonstrating their systems biology credentials. Often such claims have left observers confused about what really constitutes systems biology.

The next decade is likely to be systems biology's journey through adolescence to adulthood. Perhaps, as Pfizer's newest SB department member Carolyn Cho suggested at DDT 2007, the mark of adulthood will be the disappearance of the term altogether, as it merges into the mainstream of biology.

Systems Biology of the Cell

By: Geert Potters, Ph.D. (*Dept.of Bioscience Engineering, University of Antwerp*)

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How can we understand all the interactions between DNA, mRNA and proteins, as part of one entire network? Recently, scientists have made major steps towards answering this question.

From Gene to Genome

Over the last two decades, we have seen a shift of focus in the biomolecular sciences, from the desire to understand how a single gene functions, to understanding how all genes and gene products of a cell work together. This new paradigm for understanding interrelationships between networks of biological processes is termed systems biology, and when applied to the cell biology, it is primarily about the network of genes, mRNA and proteins. This approach to scientific analysis rests on a new way of looking at processes: understanding them within a much broader context than ever before. Consequently, this approach rests on recent technological progress that allows data to be analyzed in much larger amounts. What is the concept of systems biology of the cell — what are its roots, what are its aims, and (in a minor way) what are the technological assets it depends on? (Figure 1).

Molecular Biology Is Maturing

With the publication of Watson and Crick's landmark paper on the structure of DNA (1953), the birth of modern, molecular biology became a fact. Suddenly, a paradigm was created that gave the study of heritable traits a physical, molecular basis and made the theories of Mendel, Morgan and, yes, even Darwin, tangible. Over time, we used this knowledge to understand the structure of a gene, how it is expressed, and how a mutation affects not only a base pair, but also the concomitant protein and its functioning. Crucial steps along this way were of course the development of methods to determine the sequence of short pieces of DNA (i.e., the methods of Maxam and Gilbert 1977, and Sanger 1977), how to produce more DNA in a bacterial cell (hence, to clone a gene), and since 1980, how to amplify desired DNA sequences in vitro by way of the polymerase chain reaction (PCR) technique (Mullis *et al*. 1986, Mullis and Faloona 1987).

This approach has led to a staggering amount of data regarding individual genes. Scientists have been able to ask, and answer, hitherto unanswerable questions. On which chromosome are particular genes situated? What is their function? How and by which factors are they regulated? At the beginning of the twenty-first century, it is about time to make a new transition. Instead of focusing on the individual genes and their effects, biologists and biochemists have created an arsenal of techniques and methodologies to attack a question that is simple in itself, but which requires a lot of complex information to be answered: how is the combination of all genes within a cell able to govern all the reactions that go on in a cell? This question stems from an insight, characterized by one scientist, that "multi-scale dynamic complex systems formed by interacting macromolecules and metabolites, cells, organs, and organisms underlie most biological processes" (Vidal 2009). Yet, this is the goal of biology at the molecular level — to understand the collaborative functioning of the elements as being part of a system that forms a cell, a tissue, even an organism. Indeed, nothing in biology acts alone: everything acts in conjunction, opposition, and synergy with other elements. Genes regulate each other's activity, gene products join forces or inhibit each other, cells communicate, metabolites move from tissue to tissue, and everything is interconnected. Systems biology aims to understand this complexity.

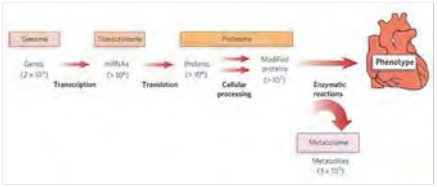

Figure 1: Systems biology allows us to think broadly.
This image shows how informational complexity increases from genome to transcriptome to proteome. The estimated number of entities of each type of molecule in a typical cell is indicated in parentheses. As an endpoint this kind of analysis, scientists might be searching for biomarkers for cardiovascular disease, so it can be detected early.

Defining Systems Biology of the Cell

In the words of Marc Kirschner (2005), "Systems biology is the study of the behavior of complex biological organization and processes in terms of the molecular constituents. It is built on molecular biology in its special concern for information transfer, on physiology for its special concern with adaptive states of the cell and organism, on developmental biology for the importance of defining a succession of physiological states in that process, and on evolutionary biology and ecology for the appreciation that all aspects of the organism are products of selection, a selection we rarely understand on a molecular level."

What does a systems biologist do? Systems biologists want to study all genes, expressed as messenger RNAs, and acting through proteins and metabolites, which play important roles in a specific cell or tissue, at a specific moment. In general, their efforts fall into four categories (defined by Kirschner 2005): quantitative measurement, creating mathematical models based on these quantitative data, reconstruction of how a cell reacts under different conditions, and the development of theories that will explain the large variation in different species in the way they react and respond to these conditions.

This goal has given rise to several new offshoots of the scientific technical subspecialties. First, there is the development of high-throughput analytical technology to be able to collect the vast array of data that systems biology requires. Fast and reliable methods have been developed to assess the different levels in a cell (the DNA or the genome, the RNA or the transcriptome, the protein pool or the proteome, and the metabolite pool or the metabolome). A few of the most important ones are discussed below. Second, there is the management of these vast sets of data, the field of bioinformatics (Rodrigo *et al*. 2008). Indeed, one of the most pressing needs emerging from this type of focus is an efficient way to store the ever growing collections of A's, T's, G's and C's (in databases such as the NCBI Genbank, or the protein-centered SWISS-PROT) which also allows for fast retrieval of data. Indeed, the amount of data that has come pouring in has grown exponentially (Boguski 1998). Apart from that, a number of applications had to be developed to facilitate a thorough analysis of these data. For example, there are analysis tools used to translate DNA to RNA and to protein sequences, identify sites for restriction enzymes to cut, and identify potential protein modifications. (See the collection at European Bioinformatics Institute, National Center for Biotechnolog Information, or Swiss Institute of Bioinformatics.) Other tools enable searches for homologous but not completely similar sequences, such as the widely known Basic Local Alignment Search Tool or BLAST (Altschul *et al*. 1990).

Moreover, there is the marriage between biology and mathematics. Using sets of differential equations, scientists try to summarize the biological data they amassed into working, robust mathematical models (Klein & Hölzel 2006, Westerhoff *et al*. 2009). In this respect, systems biology stems from earlier attempts to draw up a number of simple physical and mathematical models on what is called "biological self-organization." This approach is motivated by questions such as, how do biological structures come into existence, based on the properties of their constituent parts, and given their unique thermodynamic situation (Prigogyne 1967)? Models like those covered aspects of cell development, such as morphogenesis (Turing 1952, Ciliberto *et al*. 2003), microtubule formation (Pollard 2003), leaf phyllotaxis (Smith *et al*. 2006) or separate metabolic pathways (Polle 2001). In the future, they are bound to describe the behavior of the complete cell. Additionally, these models will allow for finely-tuned targeted manipulation of a cell's metabolism, and thus for more efficiency in genetic engineering (Goryanin *et al*. 1999, Patil *et al*. 2004).

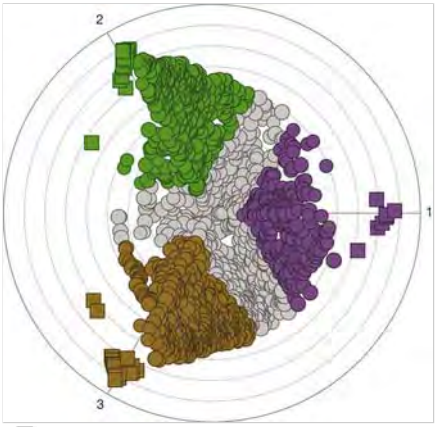

Figure 2: Scientists studied malaria-infected blood cells and observed transcription patterns of the pathogen.
Transcriptional profiles of the malaria pathogen Plasmodium falciparum were isolated from human blood samples. Transcription profiles from these plasmodia were compared to typical transcriptional profiles in yeast. Systems analysis of these expression data in these cells show that expression profiles cluster into three groups (colors). This computational analysis and comparison with previously described yeast expression profiles revealed three distinct P.falciparum physiological states characterized by glycolytic growth (purple), a starvation response (green), or an environmental stress response (brown). This improved understanding of the physiological diversity of the parasite may help to explain the range of outcomes after infection and lead to novel therapeutic approaches.

On the Horizon

Molecular biology is currently at the center of a firestorm of new questions supported by new technologies. Out of this has emerged new methodologies like genomic sequencing and whole genome comparisons, which enable us to change the way we study living cells (Figure 2). These developments are not merely new steps along the beaten track, but herald a new way of thinking about and in biology (Westerhoff & Palsson 2004). After all, until now, biological complexity could only be understood through the study of the individual parts. In molecular biology and biochemistry, the questions have always been more of a qualitative nature, such as, have we cloned the relevant gene or not? Is it present and active? In which cells? Systems biology is bound to take away that burden, to become much more quantitative, and to offer a more integrated perspective on the inner workings of a cell, without having to resort to vagueness. This makes systems biology a difficult yet exact type of science. For many biologists (and in particular molecular biologists), equations were something that belonged in physics labs, and advanced mathematics and in depth statistics were a less prevalent and optional tool, with the exception of field ecologists, for whom multivariate statistics are typically present from the start. They will have to learn to cope with equations that will at least

parallel those of advanced physics in their complexity. However, the rewards are enormous, and their magnitude is probably only beginning to surface.

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What Is Systems Biology?

Sept. 13, 2007 | Of the many systems biology definitions floating about, ISB founder Lee Hood has the clearest and most encompassing view. There are two types of systems biology, he suggests: One sets out to decipher the biological function of molecular machines (e.g., proteasome); the second seeks to identify and decipher the function of biological networks and how they "capture, transmit, integrate, and disperse biological information."

In this context, Hood argues modern systems biology requires six essential features:

1. Quantitative measurements for all types of biological information.

2. Global measurements of dynamic changes in all genes, mRNAs, proteins, etc., across state changes.

3. Computational and mathematical integration of different data types — DNA, RNA, protein interactions, etc. — to capture distinct types of environmental information.

4. Dynamic measurements across developmental, physiological disease, or environmental exposure transitions.

5. Utilization of carefully formulated systems perturbations.

6. Integration of discovery-driven and hypothesis-driven (global or focused) measurements. The systems biology cycle: perturbation-measurement-model-hypothesis-perturbation-etc. **-- J.R.**

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