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# Inorganic Signatures of Physiology: The X-Ray Fluorescence Microscopy Revolution

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## 1. Introduction

### 1.1 Metals as an essential, yet potentially toxic part of cellular chemistry

Metals are vital to nearly all the life processes within the cell. It is estimated that nearly a third of cellular proteins bind metals. Yet, the very same properties of these metal ions which make them so useful, also makes them potentially hazardous within the cell. Endogenous metals, such as copper, zinc, and iron are also potentially toxic, performing deleterious redox chemistry if not carefully controlled and regulated (Finney and O'Halloran 2003). The intricate cellular machinery that manages these metals are collectively known as the metal homeostasis and trafficking proteins of the cell. While we have learned much with respect to how the cell partitions and allocates metals, and at times attempted to define the 'concentration' in various compartments of the cell, instead we have begun to see how ill-defined a 'resting' condition really is, and how much the partitioning of cellular metals can change (Dodani, Leary et al. 2011; Qin, Dittmer et al. 2011).

What is it about metals which make them so critical, and so useful? Unlike other elements, biological metals, and particularly the first row of transition metals in the periodic table, have a partially filled d-shell of orbitals. This gives them multiple semi-stable oxidation states under ambient conditions. And the changes between these states provide reduction and oxidation potential for the chemistry of the cell (Bertini, Gray et al. 2007). For example, iron, as part of hemoglobin, shuffles between a (II) and (III) oxidation state to perform the vital act of delivering oxygen throughout the body. However, the same element, iron, if it were a 'free' aquo- ion in the cytoplasm of the cell would likely undergo Fenton chemistry, and these same changes in oxidation state would give rise to the generation of radicals that would damage the cell.

Still, biology manages, most of the time, to work. The efficient chemistry of these life processes often exceeds our own ability to accomplish their work synthetically. Unraveling the ways in which the chemistry of these metals is controlled promises not only to improve what we know about chemistry, accomplishing important reactions such as the oxidation of methane or the fixation of nitrogen in new and more efficient ways, but also to remarkably improve our understanding of biology, and our ability to manipulate it as well.

## 1.2 The location of metals, and bioavailability, within the cell regulates cell function

Metals serve roles in thousands of proteins and enzymes, and are found in various species throughout the mammalian cell. Good recent reviews of biological copper (Boal and Rosenzweig 2009; Banci, Bertini et al. 2010; Lutsenko 2010), iron (Kosman 2010), and zinc (Eide 2006; Tomat and Lippard 2010) exist. Our understanding of the location and bioavailability of metals, until rather recently, was mainly accessible by studying the properties of the proteins that bind them.

For example, one role of copper is as an important component of cytochrome c oxidase, which performs respiration in mitochondrion (Tsukihara, Aoyama et al. 1995; Tsukihara, Aoyama et al. 1996). From this as well as other examples, we know copper to be an important component in the mitochondrion. Among its many roles, zinc is a critical structural component of zinc-finger proteins, which regulate transcription in the nucleus. A significant reference of zinc-binding proteins is available for understanding the compartments of the cell where zinc may be found (Vallee and Auld 1990). Taken together, this kind of indirect knowledge leads to a coordinated, systems-based approach to understanding the location of cellular metals, as has been recently applied in the case of copper (Banci, Bertini et al. 2010).

These indirect approaches are also useful in understanding bioavailability of metals. For example, based upon the measurement of the metal-binding constant of superoxide dismutase we have been able to extrapolate that there is no free copper in the cytoplasm of yeast (Rae, Schmidt et al. 1999). Additionally, measurement of the zinc-binding potential of the CueR copper regulatory protein demonstrated that there was no 'free' copper in the cytoplasm of *E. coli* bacteria (Changela, Chen et al. 2003), setting the window of such ions at less than one atom per cell.

In addition to achieving the chemistry vital to the cell, metalloproteins open up a new avenue of cellular regulation. Not only can their activity be up- or down-regulated by changes in the expression level, but also by changes in the availability of their metal cofactors. An elegant illustration of this is the work of Tottey et al., which found, in examining the periplasm of the bacteria *Synechocystis* PCC 6803 that compartmentalization can be used to keep competitive metals out of the 'wrong' nascent proteins (Tottey, Waldron et al. 2008). In another, more clinical example, it has long been known that copper availability can modulate the growth of tumors in the body (Pan, Kleer et al. 2002). When copper is depleted by administering copper-chelating compounds such as tetrathiomolybdate to the patient, the growth of new blood vessels as well as the tumors that rely on them is inhibited.

## 1.3 We can now visualize changes in cellular metal distributions, and their signature patterns, during physiological changes

Recent technological advances have also made it possible to directly visualize metals within cells at the sub-cellular level. As early as the 1980's, advances in microanalysis were enabling the development of electron microscopy capable of compositional analysis - or the ability to distinguish the chemical composition of samples at the cellular or subcellular level. An excellent example of this is the work of Peter Ingram and Ann Le Furgey (Ingram, Shelburne et al. 1999). One of the limitations of electron microscopy is the thickness of samples, which generally must be no more than 100 nm thick, requiring specialized sample preparation.

On the other hand, hard X-ray microscopy (~10 keV or greater incident energy), generally does not face this limitation. The focal depth is on the order of 200 – 300 microns, and anything thinner than this will simply appear as 2-D projection of the volume. Thus, as zone plate technology and third generation synchrotron sources have developed, the minimal sample preparation required has made X-ray fluorescence microscopy a very accessible technique.

The accessibility of X-ray fluorescence microscopy, with relatively simple sample preparation and publically available synchrotron facilities, has facilitated its application – even to areas quite clinical and far removed from the physics of the synchrotron facilities that support it. The findings, some of which are highlighted below, have at times been startling. Could 80% of the cell's copper simply be exported during the angiogenic process of tubulogenesis (Finney, Mandava et al. 2007)? Isn't this a drastic commitment of energy? Despite clinical findings regarding chelation, as mentioned earlier, it is doubtful any scientist would have predicted this. But, it is precisely the directness of these methods which makes such surprising findings possible.

#### **1.4 Are these signatures diagnostic?**

According to the National Cancer Institute, a biomarker is “A biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition. Also called molecular marker and signature molecule”. In this sense, a biomarker is something that is diagnostic – serving as an indicator of a physiological state or disease condition.

The cellular distributions of metals clearly have the potential to fulfill this role. As we learn more about the distributions and compartmentalization of cellular metals, and how these change in various conditions, it is increasingly clear that signatures should exist.

## **2. Overview of X-ray fluorescence imaging**

### **2.1 Principles of biological X-ray fluorescence imaging**

Fluorescence exists in many familiar forms. It is the absorption of light at one wavelength, and its re-emission as light of a longer wavelength (with less energy). It can be seen when you put certain laundry detergents under a simple black-light, which you cannot see with your eyes, and watch optical light come back out, making it 'glow'. Optical fluorescence like this is also a critical tool in almost all of biology, where even the most complex optical fluorescence microscopes still use monochromatic light and emission filters to image optically-fluorescent dyes and protein labels, revealing information about cellular structures. X-ray fluorescence imaging is fundamentally no different from this. However, unlike optical light which excites vibrational states, X-rays are of such energy that, for metals, they excite the electrons bound to the atom directly.

#### **2.1.1 Basics of X-ray fluorescence**

The energy and wavelength of light are inversely related, as follows from Equation 1, where  $E$  is energy,  $h$  is Planck's constant,  $c$  is the speed of light, and  $\lambda$  is the wavelength.

$$E = h \times c / \lambda \quad (1)$$

Looking at this equation, it is simple to see that if the energy of an incoming photon is decreased, such as might happen when it runs into an electron, the photon is not only reduced in energy but its wavelength will increase, changing its 'color'. In fact, the photon may excite the electron into a higher energy state. When this happens, it leaves behind an opening, or 'hole' in the electron shell. Since the spacing of orbitals, or the difference in energy between them, is constant for a particular metal atom, when an electron 'falls' back down in energy, into the 'hole' that was left behind, it emits light at a very characteristic energy – much like a pipe of a particular length on an organ plays a very specific note. It is this property of X-ray fluorescence, the fact that each element – zinc, copper, iron – will emit fluorescence at characteristic energies with specific relative intensities that are intrinsic to the metal itself, that makes it possible to distinguish the emission spectrum of iron from that of copper, for example. Or to distinguish how much of either one is present in a mixture.

### 2.1.2 Special considerations for biological samples

Critical to the success of biological X-ray fluorescence imaging is preparation of samples which are both structurally and compositionally intact. At the same time, the samples must be preserved such that they can withstand the damaging potential of a focused X-ray beam. The most ideal way of ensuring this is to prepare samples which are frozen in a vitreous glass of ice. This avoids creating crystalline ice, which would break cellular structures and membranes and is not a simple task. Also, to keep the sample completely frozen without any recrystallization of the ice is not straight-forward. Current research is attempting to achieve both of these.

Yet, much interesting and useful research has been done on dry samples. Recent studies examine either flash-frozen and freeze-dried samples, or chemically fixed and dried samples (McRae, Bagchi et al. 2009).

Another consideration in examining biological samples is that the emission peaks of most common biological metals overlap quite strongly in the emission spectrum generated by energy-dispersive detectors. Thus, proper fitting of the data, including de-convolution of these peaks, is critical to correct assignment of intensity to a metal of interest. The development of software, particularly MAPS, has been of paramount importance in this field (Vogt 2003). Likewise, the selection and use of reference standards, to convert emitted intensity to a calculated quantity, is also critical to proper analysis.

## 3. The revolution: Peering into the unseen

### 3.1 Case 1: The biology of selenium

#### 3.1.1 Overview of selenium biochemistry

Selenium exists, in mammals, primarily as part of selenomethionine or selenocystine, and less abundantly as selenite, selenide, monomethylselenol, dimethylselenide, trimethylselenonium, L-selenomethionine (SM), Se-methyl-L-selenocysteine. Because of its chemical similarity, it is utilized by the body in many of the same pathways as sulphur. Some controversy has surrounded its use as a nutritional supplement in the prevention of cancer – where it has been purported to function in an anti-oxidative capacity.



### 3.1.2 What XFM has revealed

X-ray fluorescence has shed light on the biological roles of selenium in biochemistry. Kehr et al. obtained beautiful images, the first of their kind, of the selenium in sperm (Kehr, Malinouski et al. 2009). It had long been known that selenium is essential for sperm production and therefore fertility in mammals (Maiorino, Roveri et al. 2006). By directly imaging the selenium in the sperm at various stages of development, scientists found that a high and specific accumulation of selenium occurs during spermatid development. Further, they determined that it related to an increased need for the plasma selenoprotein SelP in order to produce additional mGPx4 protein. This work not only expanded the current understanding of selenium biology, but also demonstrated the utility of direct imaging of selenium at the subcellular level for better understanding of mammalian biology.

In another example, the effects of GPx1 deficiency were explored in mice. GPx1 is the major mammalian selenoprotein and it is expressed at a particularly high level in the liver (Malinouski, Kehr et al. 2011). The uniform distribution of Se in hepatocytes is consistent with the concept that XFM largely detects GPx1. In this work, it was found that in addition to homogenous signal from GPx1, the kidney also showed highly localized circular structures of Se surrounding proximal tubules. It was reported that this signal represents GPx3, which was secreted from these tubules and remained bound to the basement membrane. It represented approximately 20% of the Se pool in mouse kidney, and an even higher fraction in the kidney of the naked mole rat. This observation supports the postulate that the production of these two proteins, and their sources of selenium, are separate. The authors also postulate that advances in X-ray fluorescence imaging, increasing its resolution and sensitivity, will lead to a greater understanding of selenium biology.

## 3.2 Case 2: A role for zinc in cell fertilization, differentiation, lactation

### 3.2.1 A historical perspective on the cell biology of zinc

Zinc has long been known to play an important role in biology. Studies of the biochemistry of zinc may well have first begun in the 1950's, with the study of metallothionein. We now know that zinc plays both structural roles, such as in zinc finger proteins, and catalytic roles such as it does in carbonic anhydrase. Yet, there remains much to learn about this metal. Results from direct X-ray fluorescence imaging of this element in cells may indeed have revealed that there is still much to learn.

### 3.2.2 A new view of zinc from XFM

One of the first findings regarding zinc (utilizing sub-micron X-ray fluorescence imaging) was that it may be involved in cell differentiation, particularly looking at HL-60 cells (Glesne, Vogt et al. 2006). In examining the growth of human embryonic stem cells, taking a systems biology approach to examining entire colonies of cells and all the first row transition metals, we also found that the amount of zinc present in cells directly correlated with their differentiation (Wolford, Chishti et al. 2010). The images in Figure 1, of stem cells differentiated with retinoic acid, are particularly illustrative. Loss of Oct4 (pink) is associated with higher zinc (red in 'Zn' panel). This was found to be true regardless of the method of differentiation, or whether the cells at the outer edge or at the center of the colony

were the ones to differentiate first. This exciting finding brings us to a new understanding of how little we know about the majority of nuclear zinc, as well as the roles of metals during differentiation.

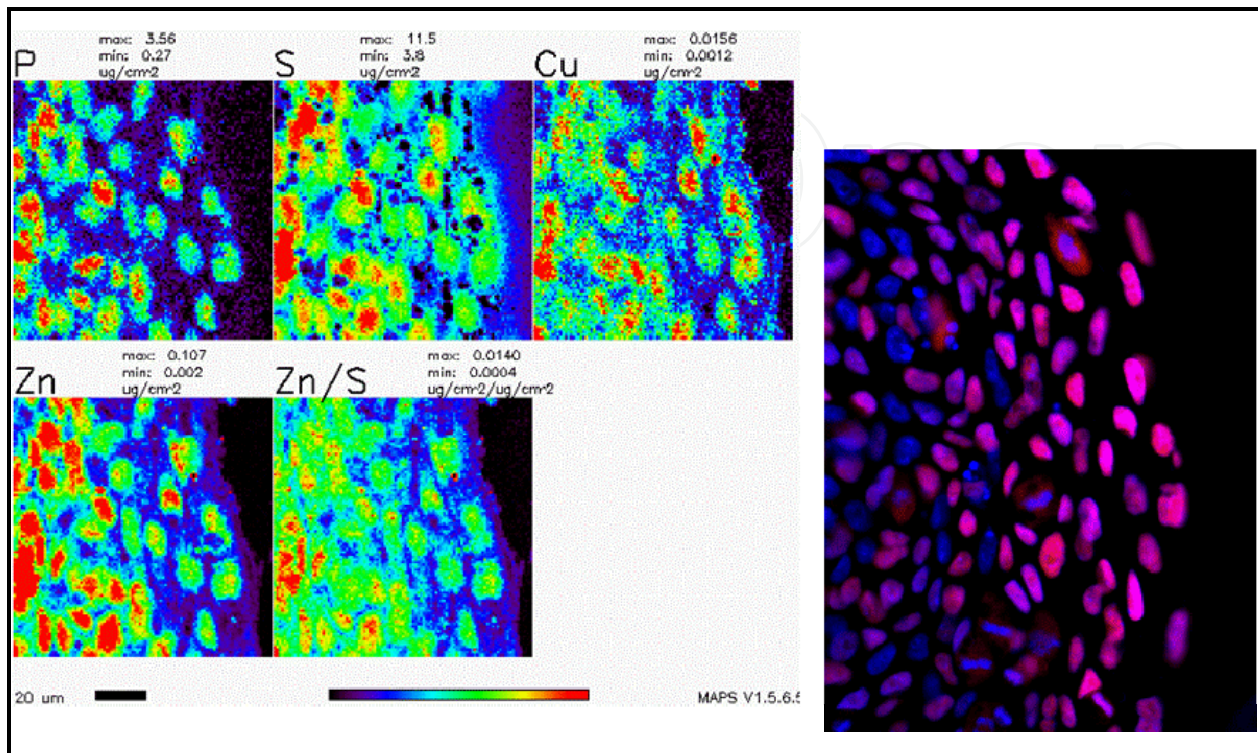


Fig. 1. Correlating XRF and immuno-fluorescence images of human embryonic stem cells.

At the same time, zinc plays many roles in the cell, as well as roles even outside of the cell. An elegant example of work utilizing X-ray fluorescence to better understand zinc physiology is reported by the Kelleher group in their studies of lactation (McCormick, Velasquez et al. 2010). In contrast to the coordination of zinc throughout much of the cell, zinc in milk is significantly associated with lower molecular weight molecules – at relatively high concentration relative to other essential metals. And it is not known how the mammary gland regulates the transfer of zinc into milk. By directly imaging the zinc in mammary tissue of both lactating and non-lactating mice, the researchers were able to demonstrate that zinc associates with a distinct peri-nuclear pool. Further, through experiments utilizing the chemical indicator Fluo-zin-3, which is a zinc indicator, together with dyes for the endoplasmic reticulum, mitochondrion, and Golgi, optical fluorescence microscopy indicated that this zinc was ‘labile’ on account with its ability to bind the zinc dye, and at least partially associated with the Golgi complex. This work represents some of the first X-ray fluorescence imaging of mammary tissue at the sub-micron scale, and suggests that the pathways for zinc export during lactation likely are similar to those utilized in the prostate.

Another particularly surprising finding from direct X-ray fluorescence imaging of metals in cells relates to fertilization. Work by a team of scientists from Northwestern University has recently shown that the accumulation of zinc is essential for fertilization (Kim, Vogt et al. 2010). In this work, single-cell elemental analysis of mouse oocytes by X-ray fluorescence microscopy revealed a 50% increase in total zinc content within the 12-14-h period of meiotic

maturation. While the reason for this is still unclear, the team has recently reported that experiments utilizing extra-cellular optically-fluorescent zinc indicators have shown that some of this zinc is exported upon fertilization (Kim, Bernhardt et al. 2011). This may be an example of the cell using metal bioavailability to regulate protein function.

### **3.3 Case 3: The potential of copper as a dynamic, signaling molecule**

#### **3.3.1 Established, enzymatic roles for copper**

Copper is widely used in biology for enzymatic chemistry. Its ability to cycle between (I) and (II) oxidation states makes it particularly useful for reduction and oxidation chemistry. It is used to activate oxygen, detoxify radicals, and in mitochondrial function. Yet, for as much as is known about copper, direct X-ray fluorescence imaging is revealing new roles, and changes in distributions that may have the potential to serve as biomarkers of the future.

#### **3.3.2 Viewing copper differently – Dramatic fluxes of copper**

It was reported in 2006 by Gitlin et al. that copper in hippocampal neurons appeared to be exported following NMDA-receptor stimulation (Schlief, Craig et al. 2005). The use of Cu-64 to try to measure this export made it somewhat difficult to determine exactly how much of the cellular copper was exported, or image exactly where the copper was. But, clearly, new roles for copper were emerging.

Shortly after this, Finney et al. reported that a dramatic efflux of copper occurs during the angiogenic process of tubulogenesis, or the process by which new capillaries are formed. As mentioned earlier, copper had long been known to be important to angiogenesis, and thus also to the growth of cancerous tumors that rely upon a growing blood supply. By directly imaging the tubulogenesis process, at fixed points, using X-ray fluorescence microscopy scientists found that between 80-90% of the cell's copper was exported at early points, and then taken back up later in the growth of capillary-like structures (Finney, Mandava et al. 2007). Exactly why this happens remains a mystery, and has sparked new efforts in the development of tools for metalloproteomics (Finney, Chishti et al. 2010). From this, one might speculate that a role for copper in intercellular signaling, of some sort, may exist.

Taking this technique, and applying it back to the same sort of systems which Gitlin et al. had examined, leads to another remarkable finding. As shown in Figure 2, a typical SH-sy5y cell, the majority of cellular copper is typically localized in the perinuclear area in neuronal cells. Upon stimulation, the copper may be seen to relocalize such that a significant increase in the fraction of cellular copper that is along the dendrites of the cell is seen. Not only can fluxes of copper be seen in hippocampal neurons, but they are dependent on calcium, and induced by extracellular stimulation (Dodani, Domaille et al. 2011). Clearly, undiscovered roles for copper as an important part of cell signaling exist. And X-ray fluorescence imaging is enabling our further understanding of them. As roles for metals such as this are further defined, they hold the potential to reveal patterns and signatures that may become the biomarkers of the future.



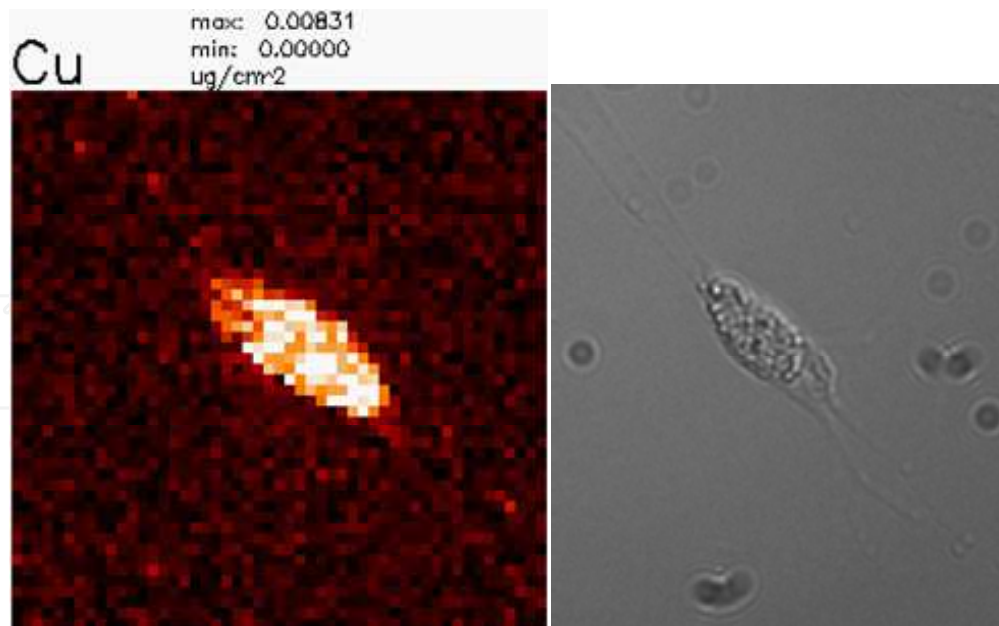


Fig. 2. Copper localizes peripherally to the nucleus in a cell

#### 4. Inorganic signatures of life: Can metal ion distributions be biomarkers of physiological state?

##### 4.1 A need to link metal ion fluxes with other biomolecules

The key to more insightful interpretations from much of this imaging is linking it to known physiological pathways. Much of what we know about cell function, and many therapeutic targets, is because of the specificity of proteins. Unleashing the potential of this tool calls for matching our developing understanding of the role of metal ions themselves in biology with our greater understanding of physiology in general.

Can the distribution of metal ions be diagnostic? Can these metal ions, or at least their signature distribution and quantity in subcellular compartments, serve as biomarkers? Consider this:

- Metal ions are essential to many processes
- Inorganic signatures can identify specific physiological processes
- Intervention, clinical diagnostics require targeting associated proteins

Thus, while it seems entirely possible that the distributions of metals may be used as a signature diagnostic of a pathological state in the future, the development of metalloproteomics and other methods of identifying their partner biomolecules is essential to therapeutic intervention in these metal-related disease processes.

##### 4.2 Need for higher resolution imaging to better define subcellular compartments

A need exists for higher-resolution X-ray fluorescence imaging, to better identify the specific cellular compartments where metals are distributed. Many examples highlighted here look at changes in metal distributions that are either 'in' vs. 'out' of the cell, 'near' or 'away' from the nucleus. At the advent of the age of nano-scale imaging of biological samples, imaging

cells at the scale of 10's of nanometers, where we can begin to see within the mitochondrion, for example, will reveal many new and exciting things about the cell biology of metals.

### 4.3 Need for other data acquisition schemes to improve statistics

Another important consideration in the context of biomarkers is speed. X-ray fluorescence imaging is currently quite slow. Samples are raster-scanned through an X-ray beam with dwell times of 1s or more per pixel. With potentially hours of scan time required for imaging a single cell, good statistical sampling is difficult to achieve. What is needed for this? Some current areas of development may help, particularly the development of fast fly-scanning data acquisition, where samples are raster scanned continuously and fluorescence information is recorded 'on-the-fly'. Another area with promise for speed is the development of microfluidic devices that will enable X-ray fluorescence spectra of whole cells to be individually captured while flowing in a stream, thus allowing measurement of at least the total metals in individual cells over populations of hundreds of cells. As techniques like these emerge, the promise of X-ray fluorescence imaging for diagnostics comes closer to a reality.

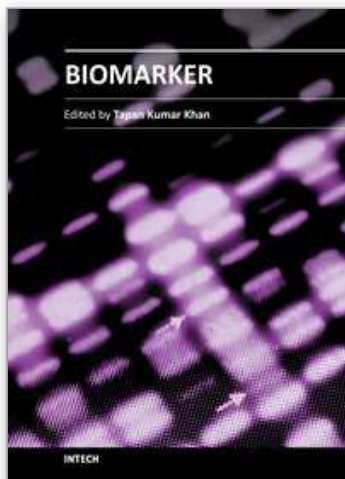
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## **Biomarker**

Edited by Prof. Tapan Khan

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Clinicians, scientists, and health care professionals use biomarkers or biological markers as a measure of a person's present health condition or response to interventions. An ideal -biomarker should have the following criteria: (I) ability to detect fundamental features of the disease, (II) ability to differentiate from other closely related diseases, (III) ability to detect early stages and stages of progression, (IV) the method should be highly reliable, easy to perform and inexpensive, and (V) sample sources should be easily accessible from body. Most of the chapters in this book follow the basic principle of biomarkers.

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