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3

Immunohistochemical Correlation of Novel Biomarkers with Neurodegeneration in Rat Models of Brain Injury

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1. Introduction

Immunohistochemistry is an important technique used to visualize specific changes in tissues as part of both normal development and pathological conditions. Immunohistochemistry (IHC) combines antibody specificity with high resolution imaging techniques which, together, can provide reliable visual evidence of presumed physiological processes. The technique is amenable to rigorous experimental design. In controlled settings, with the appropriate constraints, and image acquisition settings, data obtained becomes reliable, quantifiable and reproducible. Through the numerous past and current explorations of IHC, novel, antibody-based, 'point of care' diagnostics as well as antibody-based therapeutics will and are becoming a valuable tool for clinicians and researchers. A field that really craves such new diagnostic and therapeutic technologies is that of traumatic brain injury.

Brain injury, especially traumatic brain injury, or TBI, initiates a complex series of neurochemical signalling events. These pathological changes are mediated, at lease in part, by glutamate excitotoxity, inflammation and increased blood-brain barrier (BBB) permeability leading to numerous sequale which include neuronal hyperactivity, increased cellular vulnerability, edematous states, cellular dysfunction and consequent apoptotic and necrotic cell death. Many of these changes produce subtle and slight global manifestations and hence are invisible to current diagnostic imaging techniques such as magnetic resonance imaging or computer aided tomography scans. Recently though, the decades-long efforts of a few pioneering groups has established novel protein biomarkers for TBI (Mondello, Muller et al. 2011). Unlike many traditional biomarkers, these novel protein biomarkers can be investigated using IHC. As their discovery remains recent, the relationship between their presence in the extracellular environment and their intracellular source has not been explored.

Our group investigated one such biomarker, namely ubiquitin carboxy-terminal hydrolase-1 (UCH-L1), and found that it could provide powerful information regarding the

degenerative state of neurons. Using immunohistochemistry of serial sectioned brain slices we saw that brain areas containing degenerate neurons were devoid of immuno-signal for UCH-L1. In contrast robust UCH-L1 staining could be seen where degenerate neurons were absent. Similar IHC results were observed in surgically resected human brain tissue from patients with brain injury, although independent verification is needed. The levels seen using UCH-L1 immunohistochemistry were inversely proportional to the extracellular concentrations of this protein. In the next few sections of this chapter we will present two rodent brain injury models, IHC based cell-specific markers, chemical stains associated with neurodegeneration, the role of IHC in assessing the relationship between novel brain injury biomarker UCH-L1 and the polyanionic fluorescein derivitave, Fluorojade B (FJB), which sensitivity and specifically binds to degenerating neurons. Taken together our data suggests that UCH-L1 is lost prior to the onset of FJB target *in vivo* following brain injury

2. Rodent models of brain injury

The establishment and use of animal models of traumatic brain injury (TBI) remains vital to understanding the pathophysiology of this highly complex condition. The purpose of these experimental models is to replicate certain pathological components or phases of clinical trauma in experimental animals aiming to address pathology and/or treatment (Cernak 2005). Such models share the ultimate goals of reproducing patterns of tissue damage observed in humans. Our laboratory has extensive experience with two TBI models (Dietrich, Alonso et al. 1994; Zhou, Sun et al. 2008). In this study we explore the use of those models to assess the relationship between neurodegeneration and immunohistochemical analyses of novel biomarkers.

2.1 Acute Subdural Hematoma (ASDH)

Adult Sprague Dawley (350-400 g) rats are anesthetized with a mixture of 3% isoflurane 70% N_2O and 30% oxygen. Following determination of adequate anesthesia by monitoring toe-pinch (foot reflex-flexion to pain) and the corneal reflexes, the tail artery is catheterized to aid monitoring of vitals such as blood pressure, blood gases and pH. Detailed descriptions of the procedures have been extensively published (Daugherty, Levasseur et al. 2004; Kwon, Sun et al. 2005; Zhou, Sun et al. 2008). Briefly, the animal is intubated using a shielded 14GA i.v. catheter (BD Insyte Autoguard, 14GA 175IN, 2.1x45mm). The animal is paralyzed using Pancuronium Bromide (0.35 mg/kg every ½ hour) and connected to the ventilator. Following reduction of isoflurane level to 0.5-1.0%, the first blood gas analysis is performed to control proper ventilation of the animal. The partial oxygen tension (pO2) of ~100-150mmHg and a partial carbon dioxide tension (pCO2) of 35-45mmHg is attained before continuing with the surgery. A midline scalp incision is made to expose the sagittal and coronal sutures. A parasagittal craniotomy (4.8 mm) using a trephine (Roboz) is performed at 3.8 mm posterior to bregma and 2.5 mm lateral to the midline creating a burr hole 3 mm in diameter. Using an operating microscope the dura is incised and a blunt, pre-bent J-shaped 23-guage needle filled with ~0.4ml non-heparinized, autologous arterial blood is carefully inserted into the subdural space with the curved tip pulled up against the overlying dura; rapid-curing cyanoacrylate glue is used to seal the burr hole and secure the needle. To create the subdural hematoma 0.35 ml of the blood is slowly injected into the subdural space over 6-7 minutes. The needle is crimped and animal is treated according to a

previously assigned therapeutic intervention. As shown in the Fig.1 the hematoma is subdural and is wide spread.
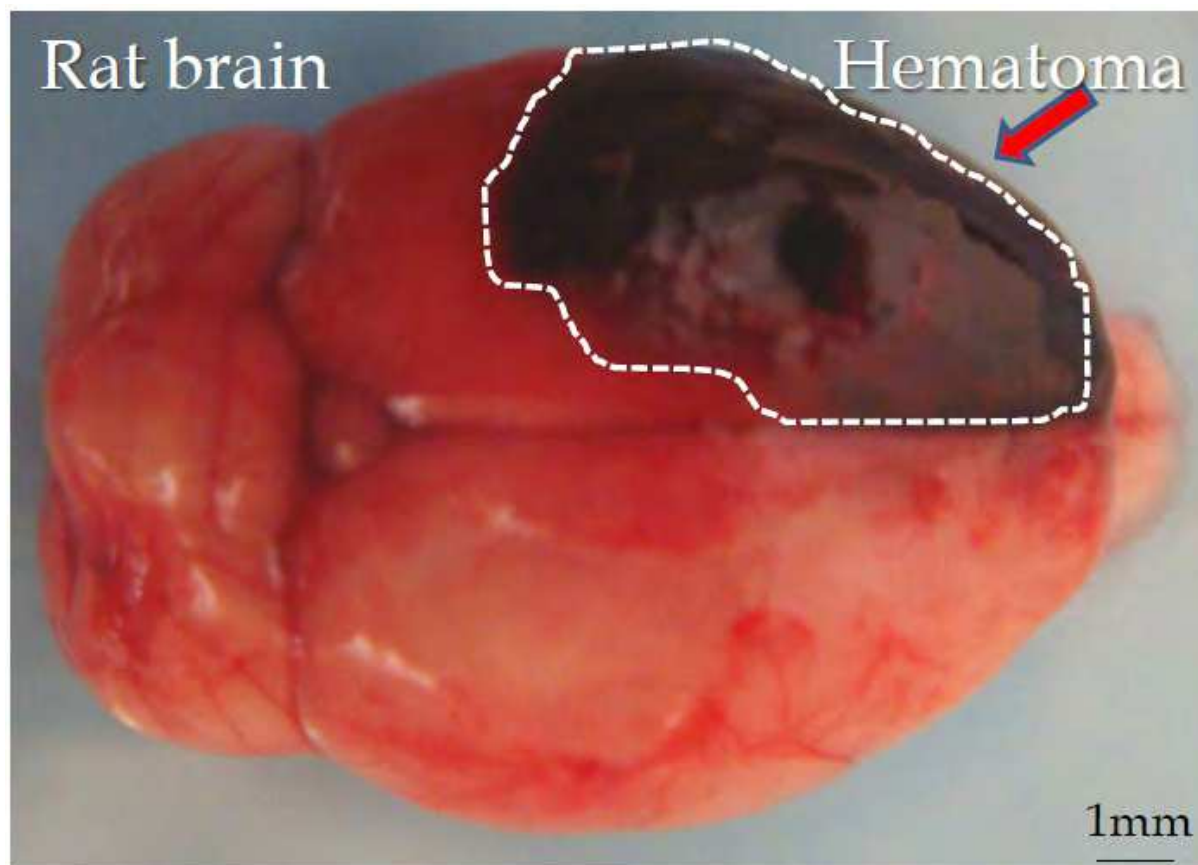

Fig. 1. Acute subdural hematoma model (ASDH) An extensive subdural hematoma (white dashed outline) can be seen on the left frontal and temporal lobe of this rat brain resulting in damage of the cortex in this area (dark area).

2.2 TBI

Adult Sprague-Dawley rats are anesthetized and a 4.8mm burr hole is made as described above in ASDH paragraph. A sterile plastic injury tube is next placed over the exposed dura and glued to the skull using acrylic adhesive. Dental acrylic is poured around the injury tube to obtain a perfect seal. After the acrylic has hardened, the scalp is closed using staples and the animals are returned to their home cage. About 18 hours after the previous preparation the rats are anesthetized, intubated, connected to a respirator and ventilated with 0.5-1% isoflurane and a mixture of 70% nitrous oxide and 30% oxygen. The animal is paralyzed with Pancuronium for mechanical ventilation to maintain arterial blood gases within normal limits. The fluid percussion (F-P) device (Custom Design and Fabrication, VCU Medical Center, University of Virginia, VA, USA) consists of a Plexiglas cylindrical reservoir bounded at one end by a rubber-covered Plexiglas piston with the opposite end fitted with transducer housing and a central injury screw adapted for the rat's skull. The entire system is filled with isotonic saline. The (aseptic) metal injury screw is next firmly connected to the plastic injury tube of the intubated and anesthetized rat. The injury is induced by the descent of a metal pendulum striking the piston, thereby injecting a small

volume of saline epidurally into the closed cranial cavity and producing a brief displacement (22 msec) of neural tissue. The amplitude of the resulting pressure pulse is measured in atmospheres by a pressure transducer and recorded on a PowerLab chart recording system (ADinstruments, CO, USA). Brain and body temperature are recorded with a thermistor into the left temporalis muscle and a rectal probe. Animals are maintained normothermic with a combination of heating lamps and a negative feedback control during the entire procedure. In this study, a moderate (1.8-2.2 atmospheres) injury was used (Dietrich, Alonso et al. 1994; Bregy 2010).

3. Cell type specific markers

Antibodies against proteins found predominantly in the central nervous system (CNS) have been successfully used to identify the CNS cells. Molecular markers for specific cell types are intricately associated with structure or function of the cells; they are also evolutionarily conserved, lending them credibility. However, changes in cell type specific markers alone are not sufficient to deduce injury effects on cells.

3.1 Neurons: NeuN

Antibodies against specific proteins found abundantly in a particular cell type can be used to identify cell types. A single clone (A60) against a vertebrate neuron-specific nuclear protein called NeuN (Neuronal Nuclei) is used to identify neurons (Mullen, Buck et al. 1992). The antibody reacts with an uncharacterized nuclear protein found in most neuronal cell types throughout the nervous system including cerebellum, cerebral cortex, hippocampus, thalamus, and spinal cord. The immunohistochemical staining occurs primarily in the nucleus of the neurons with lighter staining in the cytoplasm. The antibody cross-reacts with nervous tissue in a wide range of animals from salamanders, chicks, and rats to humans. The figure shows a confocal image tile of NeuN labeled neurons (green) in gray matter of the rat spinal cord (Fig.2). As the release of NeuN antigen into extracellular media is not characterized, the utility of this protein as a serum or cerebrospinal fluid biomarker remains unexplored.

3.2 Glia: GFAP

Glial fibrillary acidic protein (GFAP) has been identified as a brain specific protein in brains of patients with multiple sclerosis (MS), in which GFAP was purified from a large MS plaque consisting primarily of fibrous astrocytes and demyelinated axons (Eng, Ghirnikar et al. 2000). GFAP mediates astrocyte functions known to be important during regeneration, synaptic plasticity and reactive gliosis (Eng, Ghirnikar et al. 2000) (Middeldorp and Hol 2011). However, GFAP is not only expressed in astrocytes but also in the neural crest-derived non-myelinating Schwann cells (SC) and in spinal cord following injury (Gajavelli, Wood et al. 2004). Several antibodies against GFAP are widely used in research and clinically for immunohistochemical diagnosis of tumors. GFAP positive cells appear green with cytoplasmic GFAP intermediate filaments revealing underlying cellular morphology of astrocytes in an immunostained rat spinal cord section (Fig. 2). Cautious interpretation of anti-GFAP immunocytochemical results is recommended as positive staining may identify an astrocyte in the CNS but a negative result may be false. (Eng, Ghirnikar et al. 2000). Currently, GFAP is used as a biomarker for gliosis (Mondello, Muller et al. 2011).
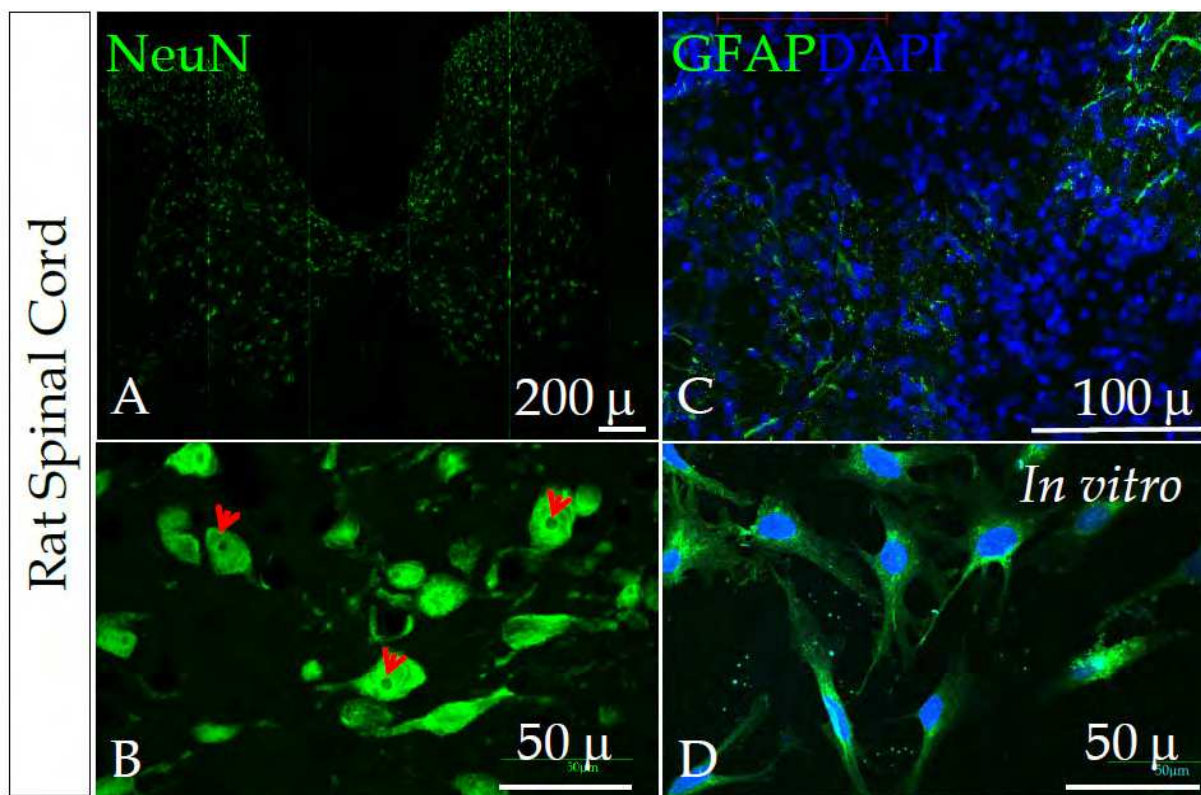
Fig. 2. Coronal sections of rat spinal cord were immunostained for NeuN (A) and GFAP (C). NeuN predominantly stains the nucleus and labels the entire gray matter. At higher magnification a few cytoplasmic processes but not nucleolus (arrowheads in B) stain with NeuN. GFAP+ astrocytes appear tluoughout the spinal cord (C). At a higher magnification GFAP-immunocytochemistry of an *in vitro* culture (rat postnatal day 4) reveals GFAP intermediate filaments underlying cellular morphology. DAPI stained nuclei (Blue).

4. Cell death markers

The standard methods for the detection of cell death in the nervous tissue include anti-Caspase-1 immunohistochemistry and chemical staining for degenerate neurons using Fluorojade B. In the following section, we investigate the co-incidence of these markers with the novel biomarkers of neural damage.

4.1 Caspase 1

A large macromolecular complex, termed the inflammasome, activates cysteine aspartic acid proteases / interleukin-1 beta converting enzyme (caspase-1). The activated caspase-1, a (p20/p10) tetramer, is necessary and sufficient for cleavage/maturation of proinflammatory cytokines interleukin-1beta (IL-1beta) and IL-18 as well as for the induction of apoptosis. Caspases inactivate enzymes, cleave structural proteins and activate other proteases in cell death. These cysteine proteases cleave after aspartic acid and can be activated through an intrinsic pathway involving the mitochondria, through an extrinsic pathway involving membrane receptors, or by an inflammatory response. Inflammation and infection cause potassium channels to open, which causes an efflux of potassium. A low level of intracellular potassium induces binding of caspase 1 to the inflammasome, which leads to

controlled inflammation. At normal potassium levels, caspase 1 is inhibited. Traumatic brain injury elicits acute inflammation including caspase-1, that in turn exacerbates primary brain damage (Mariathasan and Monack 2007; Yu and Finlay 2008; de Rivero Vaccari, Lotocki et al. 2009). Therefore, staining for these proteases can be valuable in monitoring and observing cell death and inflammation.

4.2 Fluorojade B stain degenerate neurons

Fluoro-Jade B (FJB) is an anionic fluorochrome capable of selectively staining degenerate neurons in brain tissue. It can be combined with other fluorescent methodologies, such as immunofluorescence. FJB is a tribasic fluorescein derivative with a molecular weight of 445 daltons. It has an emission peak at 550 nm, and excitation peaks at 362 and 390 nm, respectively. The exact mechanism by which FJB stains degenerating neurons is not known. Based on the affinity of the acidic FJB, it can be inferred that a degenerating neuron presumably expresses a strongly basic molecule. FJB is fairly resilient, showing no signs of fading during storage at room temperature for up to 2 years and when fading does occur, it does so in cells and background at the same rate (Schmued, Albertson et al. 1997). For the FJB staining procedure the brain sections air dried at 45-50⁰C for 30-60 minutes, rehydrated using successive 5 minute rinses in 100%, 75%, 50%, and 25% ethanol followed by 3 minutes in distilled water, placed in 0.06% potassium permanganate for 15 minutes followed by 2 minutes in distilled water, and then stained in 0.0006% Fluoro-Jade B solution in 0.1% acetic acid for 30 minutes. The slides are air-dried, immersed in xylene and coverslipped with cytoseal.

5. Biomarkers

In medicine, a biomarker is a term used for any molecule that can be an indicator of a particular disease or state of that disease. More specifically, a biomarker indicates a change in expression or state of a protein that correlates with the risk or progression of a disease, or with the susceptibility of the disease state to a given treatment. Biomarkers have characteristic biological properties that can be detected and measured in parts of the body like the blood or tissues. Although the term biomarker is relatively new, biomarkers have been used in pre-clinical research and clinical diagnosis for a long time. The most widely accepted serum biomarkers are creatine kinase isoenzyme MB (CK-MB) and troponin, which have surpassed the electrocardiogram as the standard criterion for the diagnosis of myocardial infarction (Lewandrowski, Chen et al. 2002). Other examples are glucose for the diagnosis and follow up for diabetes, cholesterol values as a biomarker and risk indicator for coronary and vascular disease, C-reactive protein as a marker of inflammation and the prostate specific antigen (PSA) as a marker for prostatic cancer between many other known biomarkers. The use of biomarkers to asses brain injury has been an area of research since late 1970s and early 1980s (Thomas, Palfreyman et al. 1978; Papa, Akinyi et al. 2010). Development of a useful biomarker of brain injury, however, has proven to be more difficult than development of biomarkers for other organ systems for several reasons. Perhaps most important, the brain is a more complex and less homogenous organ and different types of injury can occur to different types of brain cells with variable degrees of severity. In addition, the presence of a blood-brain barrier limits the amount and size of markers that can be detected in blood. Although evaluating the use of serum biomarkers to asses brain

injury has been an active area of research for more than 20 years, interest in the use of serum biomarkers to assist in the prediction of outcome after TBI is more recent, with the first studies being published in the early 1990s.
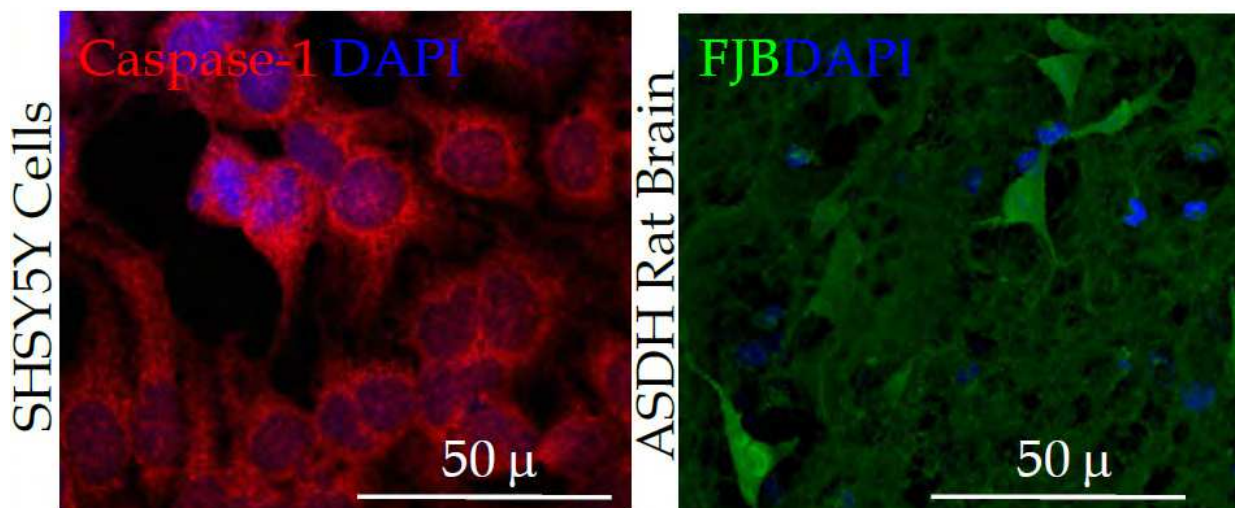

Fig. 3. A confocal image of SHSY5Y neuroblastoma cell line immunostained for Caspase-1 (red) shows cytoplasmic expression (left). Fluorojade B stained degenerate neurons appear fluorescent green in an acute subdural hematoma brain section (right). DAPI stained nuclei (blue). Magnification is shown below.

5.1 Traditional biomarkers

For primary and secondary injury in TBI several classes of biomarkers have been investigated. They include proteins, lipids, metabolites of neurotransmitters, second messengers, ions and glycolytic intermediates. These molecules are cell components that relate specifically with a neurological disease and can be found in body fluids such as cerebral spinal fluid, blood and urine. The presence of the biomarker in these fluids and tissues is detected by antibody-based assays to assess the extent of injury/disease and determine recovery. Several examples of neurological diseases and biomarkers have been reported in the literature such as β-amyloid protein, Tau protein and phosphorylated tau protein in CSF of patients with Alzheimer's disease; or abnormal accumulation of α-synuclein bound to ubiquitin inside the neurons forming Lewy bodies in Parkinson's disease.

Several traditional biomarkers, discovered by top-down approaches (known molecules with functional associations with normal brain or pathology) such as S100β, neuron specific enolase (NSE), tau, myelin basic protein (MBP) are plagued with lack of sensitivity or specificity, thus limiting their clinical utility (Dash, Zhao et al. 2010).

5.2 Novel biomarkers

A more radical bottom-up approach (in which the body fluids of injured patients are screened for molecules and their role/presence in CNS is ascertained later) has recently been developed (see Fig. 4) with advances in molecular biology, specifically in proteomics (Kobeissy, Sadasivan et al. 2008). Despite previous discoveries such as C-reactive protein
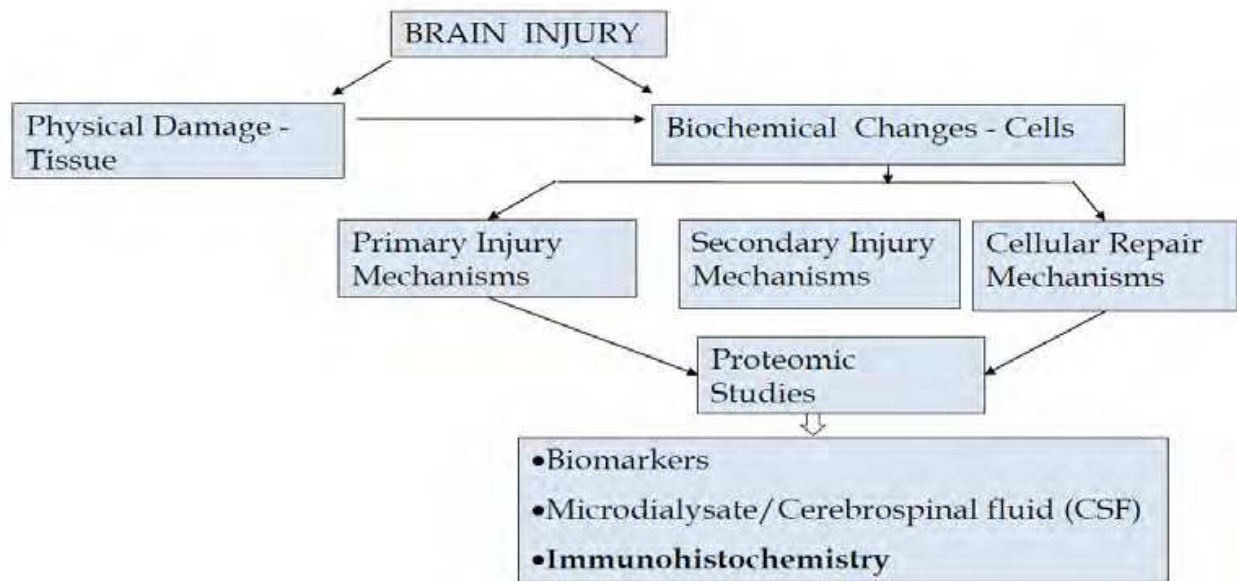
Fig. 4. Benefits of biomarkers: Diagnosis - Prognosis - Therapeutic (Diagram modified from Larner SF., 2008).

and serum amyloid A, which are not very useful as biomarkers due to lack of specificity, some progress has been made by this approach (Dash, Zhao et al. 2010). Pooled naive and injured cortical samples (48 h post injury; rat controlled cortical impact model) were processed and analyzed using a differential neuroproteomics platform. The results included 59 differential protein components of which 21 decreased and 38 increased in abundance after TBI. Proteins which decreased abundance included collapsin response mediator protein 2 (CRMP-2), glyceraldehyde-3-phosphate dehydrogenase, microtubule-associated proteins MAP2A / 2B and hexokinase. Conversely C-reactive protein, transferrin, breakdown products of CRMP-2, synaptotagmin, and αII-spectrin were found to be elevated after TBI (Ottens, Kobeissy et al. 2006). One of the novel biomarkers that increased was Ubiquitin C-terminal hydrolase-L1 (UCH-L1) (Kobeissy, Sadasivan et al. 2008), also known as neuronal-specific protein gene product 9.5 (PGP9.5). UCH-L1 was previously used as a histologic marker for neurons because of its high abundance and specific expression in neurons (Fig. 5). It is present in almost all neurons and averages 1–5% of total soluble brain protein. There are three related enzymes of this class (UCH-L1, UCH-L2 and UCHL3), but only UCH-L1 is highly enriched in the central nervous system. These enzymes are involved in either the addition or removal of ubiquitin from proteins destined for metabolism via the ATP-dependent proteasome pathway (Kobeissy, Ottens et al. 2006; Gong and Leznik 2007). To assess the reliability of UCH-L1 as a potential biomarker for traumatic brain injury (TBI), this study compared cerebrospinal fluid (CSF) levels of UCH-L1 from adult patients with severe TBI to uninjured controls and examined the relationship between these levels and severity of injury, complications and functional outcome. UCH-L1 levels in CSF were assessed using an ELISA in a prospective case control study conducted with 66 patients. Forty one patients with severe TBI, defined by a Glasgow coma scale (GCS) score of <8, who underwent intraventricular intracranial pressure monitoring were compared to 25 controls without TBI requiring CSF drainage for other medical reasons. Ventricular CSF was sampled from each patient at 6, 12, 24, 48, 72, 96, 120, 144, and 168 hrs following TBI. Injury

severity was assessed by the GCS score, Marshall Classification on computed tomography and a complicated post injury course. Mortality was assessed at 6 wks and long-term outcome was assessed using the Glasgow outcome score 6 months after injury. TBI patients had significantly elevated CSF levels of UCH-L1 at each time point after injury compared to uninjured controls. Overall mean level of UCH-L1 in TBI patients was 44.2 ng/mL (±7.9) compared with 2.7 ng/mL (±0.7) in controls (p <.001). There were significantly higher levels of UCH-L1 in patients with lower GCS scores at 24 hrs, in those with post injury complications, in those with 6-wk mortality and in those with a poor 6-month dichotomized Glasgow outcome score. These data suggest that this novel biomarker has the potential to determine injury severity in TBI patients. (Hans, Born et al. 1983; Lewandrowski, Chen et al. 2002). However, one question regarding the origin of the biomarker remained unexplored due to the nature of subjects in the study.

Fig. 5. UCH-L1 staining in normal human brain tissue. Intense brown UCH-L1+ neurons (yellow arrows) can be seen scattered throughout the section.

6. Immunohistochemistry (IHC)

Immunohistochemistry involves application of antibody to antigen-containing tissue and visualization of the antigen-antibody complex using either chromogenic or fluorescent readouts. The data can be collected using appropriate microscopy. Many of the details that are important for a successful immunohistochemical experiment have been extensively discussed elsewhere (Day and Thompson 2010) and in this book. Amplification of the signal is possible in IHC with both chromogenic and fluorescent readouts. The example for chromogenic readout includes the potentially carcinogenic 3-3'diaminobenzidine (DAB) (brown reaction stains in Fig, 6 & 9). With the advent of the tyramide signal amplification (TSA) it has been possible to obtain amplification of fluorescent signals (Raap 1998)(Fig. 6).
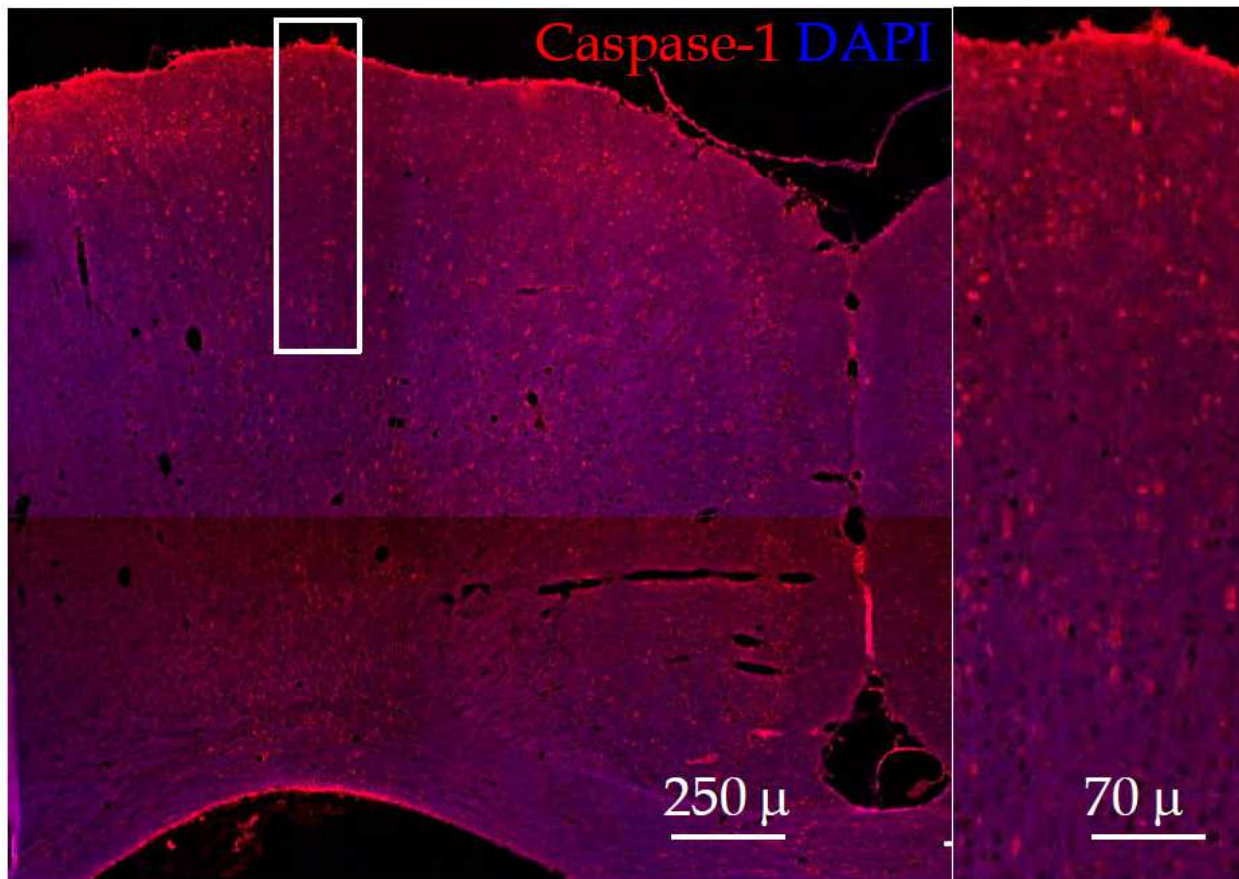
Fig. 6. Caspase-1 immunopositive cells (red) developed using TSA can be seen in cortex 24h following TBI (left). The area in the rectangular box is shown at higher magnification (right) to appreciate the cytoplasmic staining. The micron bar units are shown at the bottom of the image.

7. Factors influencing IHC

In this section we will consider a few factors that influence IHC, specifically in rodent and human brain tissue. More often than not brain tissue for IHC has to be fixed, which involves formation of novel covalent bonds between the amino acid side chains of proteins with aldehydes in order to stabilize the proteins. Fixed tissue is resistant to degradation by bacteria and fungi. Fixation of the brain is achieved by perfusion of the animal with saline, followed by 4% paraformaldehyde (PFA). Following perfusion the brain is rapidly removed and post-fixed in PFA for 6-8h. Excessive time (greater than 6-8h) in PFA can result in increased fluorescent background, which would impede use in IHC. Following post-fixation, brains are cryopreserved in 25% sucrose in phosphate buffered saline. The cryopreserved brains are placed in a Lucite/steel mold which enables accurate sectioning of the brain in blocks. Reproducibility of experiments depends on accurate blocking. The ~23mm brain is sectioned in 8mm blocks A, B, and C. Block B encompasses the hippocampus, the region of interest known to be most vulnerable to TBI. The entire block B can be embedded using media such as embedding matrix EM1 (Thermo) and sectioned on a cryostat, embedded in paraffin wax and sectioned on a microtome, or embedded in gelatin and sectioned on a Vibratome. Each of the embedding strategies is optimal for certain

antigens. Tissue sectioning can be achieved in series (which is tedious, but preserves section order), free floating (relatively less tedious but information regarding location is lost). We employed the series strategy to ensure that adjacent sections can be used for different purposes such as chemical and immunostaining. The two series can then be compared and be used to deduce relevant information. The introduction of antigen retrieval has enabled immunohistology to become an integral component of morphologic diagnosis. Antigen retrieval phenomenon involves a Mannich reaction, which occurs with the cross-linking of some proteins. Such cross-linkages can be hydrolyzed by heat or alkalis so that the process of antigen retrieval may be the simple removal of such cross-linked proteins that are sterically interfering with the binding of antibodies to linear protein epitopes in the tissue section. (Leong and Leong 2007). It is important to pay attention to the method of antigen retrieval and to match it with that of the tissue sectioning. For example, formalin fixed paraffin embedded (FFPE) sections can be subjected to antigen retrieval with a citrate buffer of pH6.0, however this buffer is not suitable for paraformaldehyde fixed gelatin embedded tissue, it requires an alkaline pH 9.0 (Shi, Shi et al. 2011). These considerations play a vital role in how the IHC turns out. Double staining by alkaline phophatase and anti-alkaline phosphatase (APAAP) in combination with bromodeoxy uridine (BrdU) and surface antigen with same isotype or different chromogenic reagent combination have aided visualization of antigens that are exclusively distributed (Chaubert, Bertholet et al. 1997). Antibodies should be applied in a manner that minimizes the amount required to achieve the antigen-antibody interaction. We routinely employ the Sequenza cover plate technology (Shandon). Briefly, the brain section containing glass slides are subjected to antigen retrieval and sandwiched by coverplates. The slides are washed and antibody applied in a 120μl volume per slide. The usual methods of acquiring images use a microscope and generate huge image files that are cumbersome to share. Recent developments have allowed digital pathology, which represents an electronic environment for performing pathologic analysis and for managing the information associated with this activity. The utility of digital pathology has already been demonstrated by pathologists in several areas including consensus reviews, quality assurance (Q/A), tissue microarrays (TMAs), education and proficiency testing. The utilization of these tools will be essential for neuropathologists to continue as leaders in diagnostics, translational research and basic science in the 21st century (Guzman and Judkins 2009). The human adrenal glands stained for a low-affinity nerve growth factor were developed with DAB and slide imaged using a Zeiss MIRAX digital slide scanner (Fig 7). Another digital slide system, Aperio, is also widely used to generate and share slides.

8. Immunohistochemistry of biomarkers

In an unpublished study, we investigated the levels of the novel biomarker UCH-L1 in ASDH model. In this study, we present data that suggests the co-incidence of chemical stain for degenerate neurons i.e., FJB and loss of UCH-L1 immunoreactivity in the tissue. By combining the aforementioned methods we found that parts of the brain that contain the degenerate neurons could be the source of novel biomarker in the extracellular fluids. In this study FJB+ cells following ASDH could be observed in the sub-cortex and cornu ammonus 1 (CA1) region of the hippocampus. To determine that the FJB+ cells were indeed degenerate neurons, the brain sections were double stained with NeuN first followed by FJB (Fig. 8).
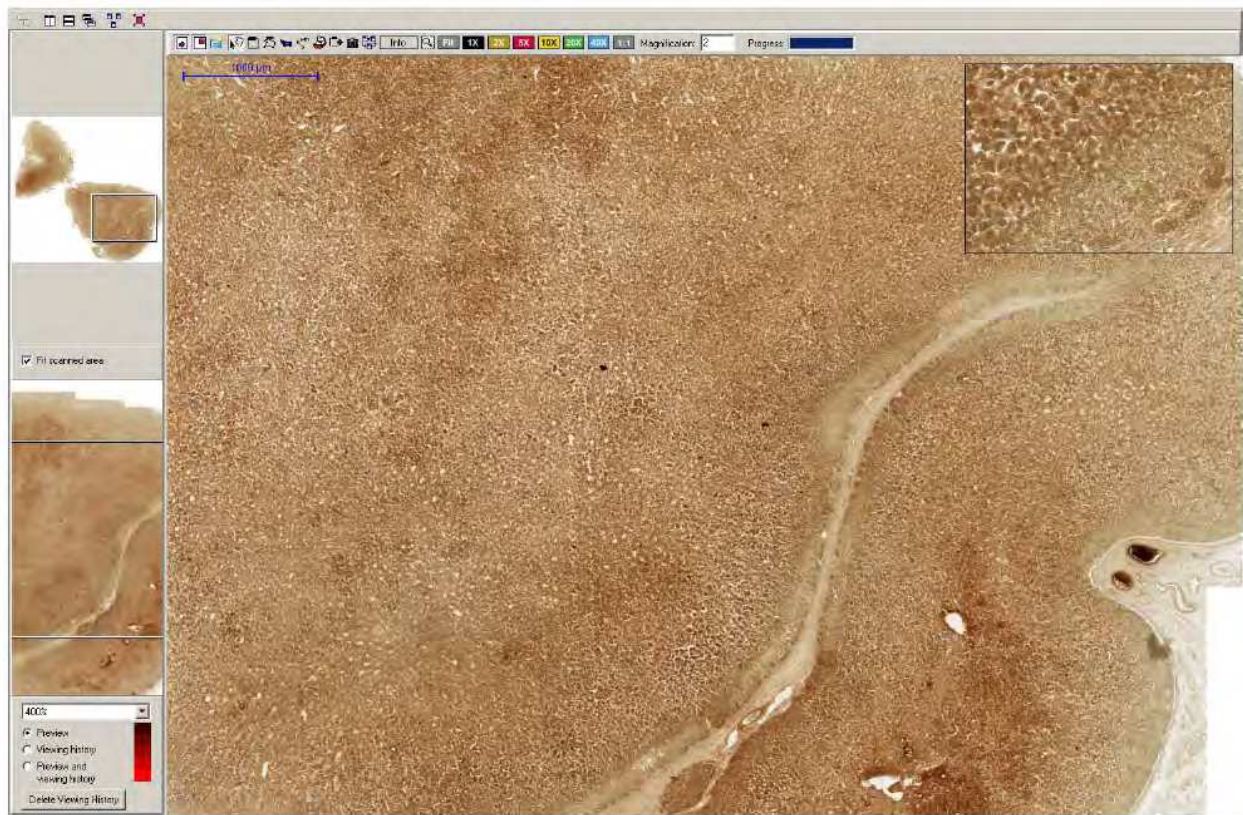
Fig. 7. Digital slide generated by Zeiss Mirax shows the entire slide (top left), the area shown in progressively higher magnification left to right. The scale bar shown on the top is 1000 µm. Brown p75⁺ adrenal medulla can be seen in the highest magnification (top right). http:/ /www.zeiss.de/mirax.

Fig. 8. FJB⁺ cells are degenerate NeuN⁺ neurons. FJB+ cells (left) double stained with NeuN (middle). Double stained cells appear yellow in the merged image (left). Absence of nuclear counter stain DAPI (blue) in FJB⁺ cells indicates neuronal degeneration (yellow arrow). All images are at same magnification, the micron bar units are shown at the bottom.

The degenerate neurons appear yellow upon merging of the confocal images. However this strategy was not possible with UCH-L1, perhaps because FJB protocol is harsh on the UCH-L1 antigen-antibody complex. To circumvent that issue, the FJB staining and UCH-L1 staining were performed on adjacent slides. First, a series of sections were stained for FJB, the distribution of the FJB cells was determined. Next, a few of the adjacent sections were double stained with FJB and NeuN. In this staining the NeuN was developed with TSA (red

fluorescence) and followed by FJB (green fluorescence). Despite the harsh conditions in the FJB protocol (with regard to antigen antibody complex stability), the NeuN+FJB+ cells appear yellow due to colocalization of the red and green signals (Fig. 8). We looked for UCH-L1 staining in regions containing FJB+ cells and those devoid of FJB. We observed that the FJB+ area was devoid of UCH-L1 staining; in contrast, robust UCH-L1 staining was present in brain regions that were negative for FJB (Fig. 9). The combination of IHC with serial sectioning made it possible to conclude that the degenerate neurons must be the source of the UCH-L1. Kinetic studies of UCH-L1 release would address the exact sequence of events that are responsible for the appearance of this biomarker in body fluids and its role in the onset of neurodegeneration.

9. Classifications of IHC guidelines

As the applications of IHC crossover into nontraditional areas such as intraoperative neurosurgery, it is important to keep the previously developed guidelines for IHC in perspective in order to avoid mistakes. The Food and Drug Administration (FDA) has guidelines for the industry regarding good guidance practices on conducting unambiguous IHC experiments, reporting IHC data (George 2009), they are summarized below.

Class I IHC's (General Controls). Class I provide the pathologist with adjunctive diagnostic information that may be incorporated into the pathologist's report, but is not ordinarily reported to the clinician as an independent finding. These IHC's are used after the primary diagnosis of tumor is made by conventional histopathology using nonimmunologic histochemical stains such as hematoxylin and eosin.

Class II IHC's (Special Controls/Guidance Document). Class II are intended for the detection and/or measurement of certain target analytes by immunological techniques in order to provide prognostic and predictive data that are not directly confirmed by routine histopathologic internal and external control specimens. The IHC's provide the pathologist with diagnostic that is ordinarily reported as independent diagnostic information to the ordering clinician, and the claims associated with these data are widely accepted and supported by valid scientific evidence.

Class III IHC's (Premarket Approval). These IHC's do not meet the criteria for class I or II, or are IHC's that meet those criteria but raise new issues of safety and effectiveness. Examples are markers used to identify new target analytes in tissues that are claimed to be clinically significant genetic mutations and that cannot be confirmed by conventional histopathologic internal and external control specimens.

Similarly, the Canadian Association of Pathologists recommendations are quality control, risk-assessment, and quality assurance (Torlakovic, Riddell et al. 2010). The aforementioned guidelines have not been set primarily for neurosurgery. With the application of intraoperative immunohistochmical analyses (Uzuka, Aoki et al. 2011) in brain tumor surgeries, virtual biopsy in brain injury diagnosis, there is a need to explore the guidelines for acceptable IHC standards for TBI neurosurgery. Uzuka *et al.*, describe methods and four successfully diagnosed cases. The time for rapid histological diagnosis was 70 minutes. Uzuka *et al.*, suggest that immunohistochemical examination is indicated under the following conditions: (1) preoperative radiologic differential diagnosis includes both high- and low-grade tumors, (2) intraoperative assessment is necessary to determine the extent of

excision, and (3) quick and accurate pathological diagnosis is necessary for early initiation of treatment after surgery. In mild TBI cases such as chronic traumatic encephalopathy (CTE), the utility of novel biomarkers such as UCH-L1 is under investigation. Application of robust IHC techniques could revolutionize the brain imaging field. The virtual biopsies could be extended to accurately diagnose mild, moderate and severe TBI. Such improvements could aid assessment of the therapeutic intervention efficacy. Currently, the molecular beacon-based technology is available in oncology to image cancer tissue in live animals (Bhojani, Ranga et al. 2011).
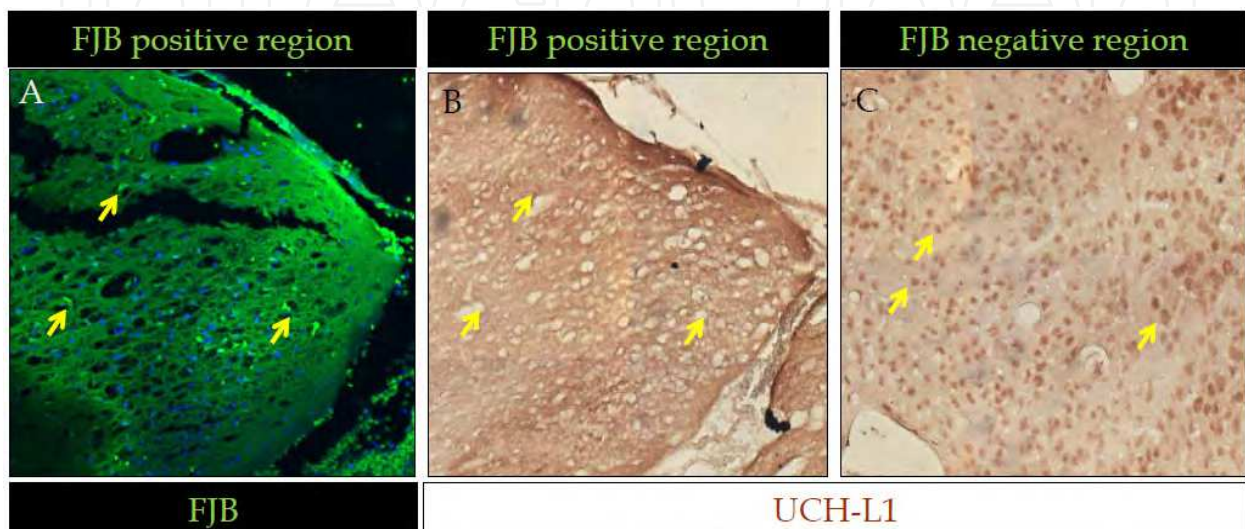
Fig. 9. A confocal image brain section from an ASDH rat shows presence of FJB+ cells (yellow arrows) in microvacuolated tissue (A). A light microscopy image of adjacent slide stained with UCH-L1 (brown) shows absence of immtmoreactivity in the FJB+ region (B). III contrast, robust UCH-L1 immunoreactivity can be seen in FJB negative region within the same section (yellow arrows) in (C). All images are shown at 100x magnification.

Brain-injury homing probes based on their affinity to the novel biomarkers would enhance visualization of the injured area. In silico structure-based drug screening using human UCH-L1 crystal structure data and virtual compound libraries identified one that potentiates the hydrolase activity of UCH-L1, and six that inhibit the activity in enzymatic assays. These compounds may be useful for research on UCH-L1 function, and could lead to candidate therapeutics for UCH-L1-associated diseases (Mitsui, Hirayama et al. 2010).

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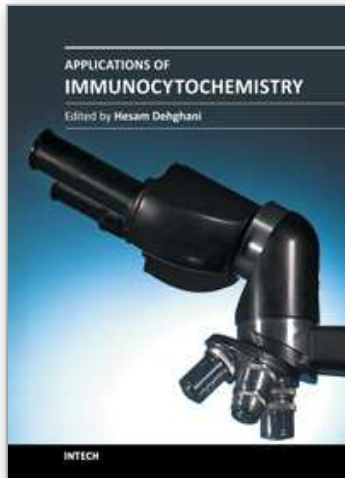
Applications of Immunocytochemistry

Edited by Dr. Hesam Dehghani

Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. However, over the years many aspects of this procedure have evolved within a plethora of experimental setups. There are different ways to prepare a given specimen, different kinds of antibodies to apply, different techniques for imaging, and different methods of analyzing the data. In this book, various ways of performing each individual step of immunocytochemistry in different cellular contexts are exemplified and discussed. Applications of Immunocytochemistry offers technical and background information on different steps of immunocytochemistry and presents the application of this technique and its adaptations in cell lines, neural tissue, pancreatic tissue, sputum cells, sperm cells, preimplantation embryo, arabidopsis, fish gonads, and Leishmania.

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