

Multiplexed Immunoassays for Brain Injury Markers

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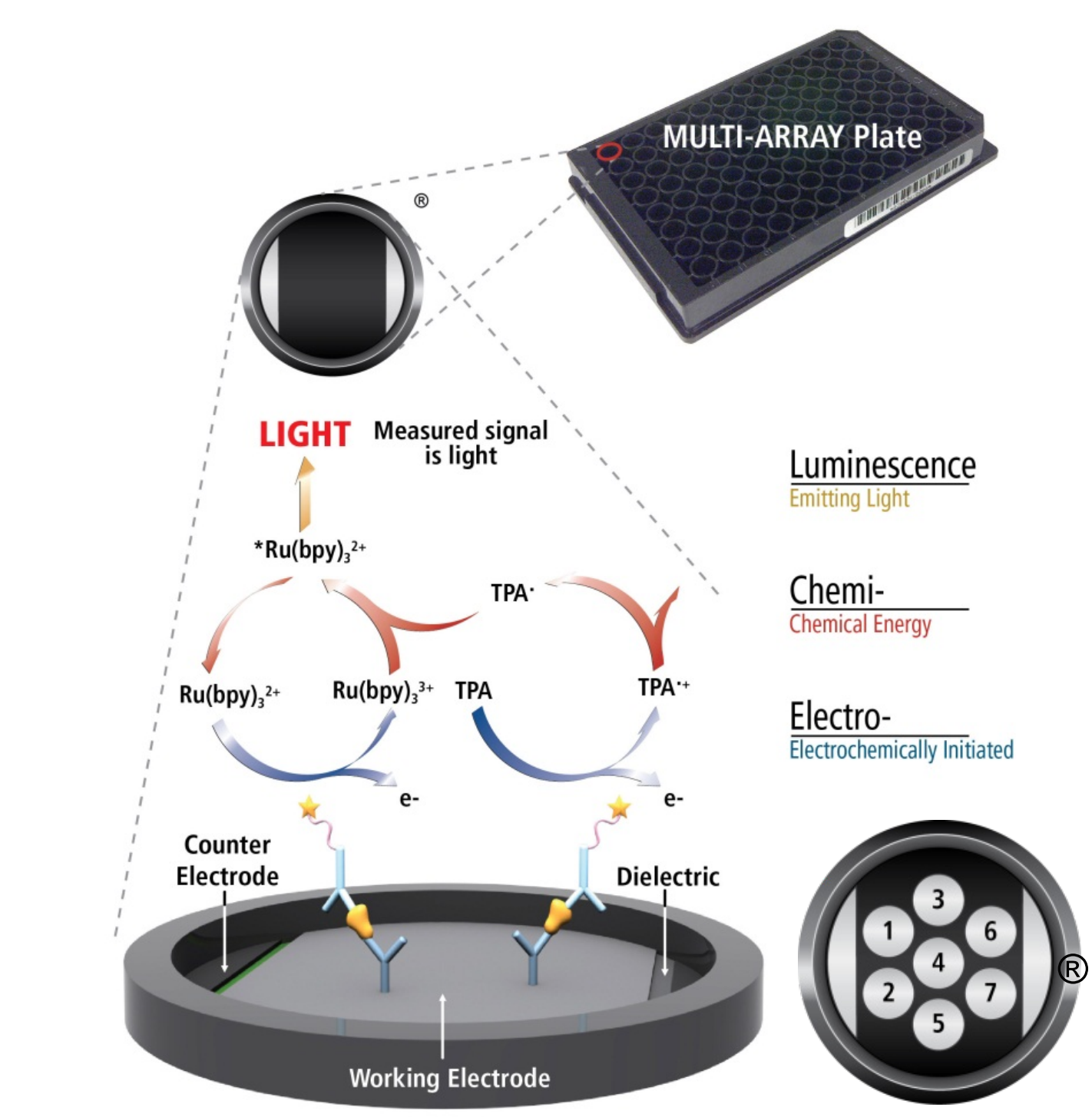
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1 Abstract

The development of effective therapies for traumatic brain injury (TBI) has been hindered by the lack of biomarkers to measure injury severity, identify subtypes of pathology, and provide evidence of therapeutic effect. Our goal was to develop a panel of TBI-related blood biomarker assays to help support research in this field. Multiplexed immunoassay panels were developed to measure plasma levels of 12 TBI-related biomarkers representing neuronal injury, astrocyte activation, vascular injury, and inflammation. The panels included Tau, GFAP, S100β, UCH-L1, BDNF, NSE, MMP-9, CKBB, NRGN, vWF, MCP-1 and VILIP-1. The assays were performed in 96-well plates using MSD’s MULTI-ARRAY® technology with sensitive electrochemiluminescence detection. The assays were qualified through analytical testing and by measuring biomarker levels in normal plasma and archived TBI sample sets (mild, moderate and severe). Some biomarkers were detectable in normal plasma while others were detectable only after TBI. Proper sample processing was important for accurate measurements, as some of the markers were also present in blood cells. In summary, a set of sensitive, multiplexed immunoassays for TBI-related biomarkers has been developed that should serve as efficient tools for investigating TBI.

2 Methods

MSD’s electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT® microplates.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

General Assay Protocols

One Incubation Step:

1. Wash plate with PBS-T.
2. Add 25 µL of assay diluent containing detection reagents and 25 µL of neat plasma, diluted plasma, or Calibrator.
3. Incubate for 1 hour at room temperature (RT) with shaking.
4. Wash with PBS-T. Add 150 µL of Read Buffer T. Read on an MSD® imager.

Two Incubation Steps:

1. Wash plate with PBS-T.
2. Add 25 µL of assay diluent and 25 µL of neat plasma, diluted plasma, or Calibrator.
3. Incubate for 1 hour at RT with shaking.
4. Wash with PBS-T. Add 25 µL of detection antibody solution.
5. Incubate for 1 hour at RT with shaking.
6. Wash with PBS-T. Add 150 µL of Read Buffer T. Read on an MSD imager.

Clinical Samples

1. Healthy & Pre-Analytical. Healthy controls were obtained from a commercial vendor as K₂EDTA plasma prepared using standard procedures (≥ 2,000 g for ≥ 20 minutes) and as K₂EDTA whole blood. A subset of samples were processed with low centrifuge speeds (200 g for 15 minutes) to be platelet-rich. All samples were frozen.

2. Mild TBI. Mild TBI plasma samples from 75 patients were obtained from the Center for Neuroscience and Regenerative Medicine’s biorepository. K₂EDTA blood samples were collected at civilian hospitals 24 – 48 hours after injury; comprehensive neuropsychological assessment and neuroimaging were collected for each patient. The majority of patients had a Glasgow Coma Scale (GCS) of 13 – 15 upon admission. Plasma was prepared using standard procedures (1,600 g for 15 minutes) and frozen. Samples from healthy controls were collected in the study and processed identically to the TBI samples.

3. Moderate and Severe TBI. Blood samples were obtained upon admission (within an average of 2 hours post injury) from TBI patients at St. Michael’s Hospital, Toronto ON. TBI was classified using a combination of neuropsychological testing and imaging. Plasma was prepared using standard procedures (1,600 g for 15 minutes) and frozen. Samples from healthy controls were collected in the study and processed identically to the TBI samples.

3 Assay Development

Immunoassays for TBI biomarkers were developed with MSD’s MULTI-ARRAY technology as part of a Department of Defense (DoD) funded program. The 12 assays discussed here represent biomarkers that have been linked to brain injury in the literature; some are well-known and others have been described more recently as potential TBI markers (e.g., NRGN, Tau, and VILIP-1). These prototype assays were developed to support testing of large sample sets for the purpose of identifying optimal biomarkers for TBI assessment.

During development, multiple antibodies were screened for each assay to identify pairs that provided the greatest separation between biomarker levels in samples from healthy individuals and TBI patients. Consideration for the form of the circulating biomarker was taken into account when appropriate; for example, anti-GFAP antibodies were chosen that recognized break-down products as well as full-length GFAP. Once antibody pairs were chosen, assays were optimized for sensitivity.

Assays that shared similar formats and sample dilution requirements (neat, 5x, or 1,000x diluted) were then grouped into multiplexes. Other factors dictating which assays were multiplexed included low cross-reactivity between assays and assay reagents, and maintaining low background signals for assays that required the highest sensitivity.

Calibrators were produced from recombinant proteins or proteins purified from human tissue. Testing was performed using an 8-point calibration curve (one zero level) run in each plate along with an analytical control sample made from normal plasma spiked with Calibrators.

All 12 biomarker assays can be measured in duplicate using less than 200 µL of plasma.

4 Assay Characterization

Dilution Linearity. Four individual plasma samples from healthy donors were diluted 2X, 4X, and 8X with Calibrator diluent. Average recovery over the three dilutions is shown for each sample along with an overall average across all samples. Samples used for GFAP, CKBB, VILIP-1, NRGN, and Tau dilution linearity were first spiked with Calibrator to provide measurable levels.

Spike Recovery. Four individual samples from healthy donors were spiked with three levels of Calibrator. Average recovery for the three spikes is shown for each sample along with an overall average across all samples.

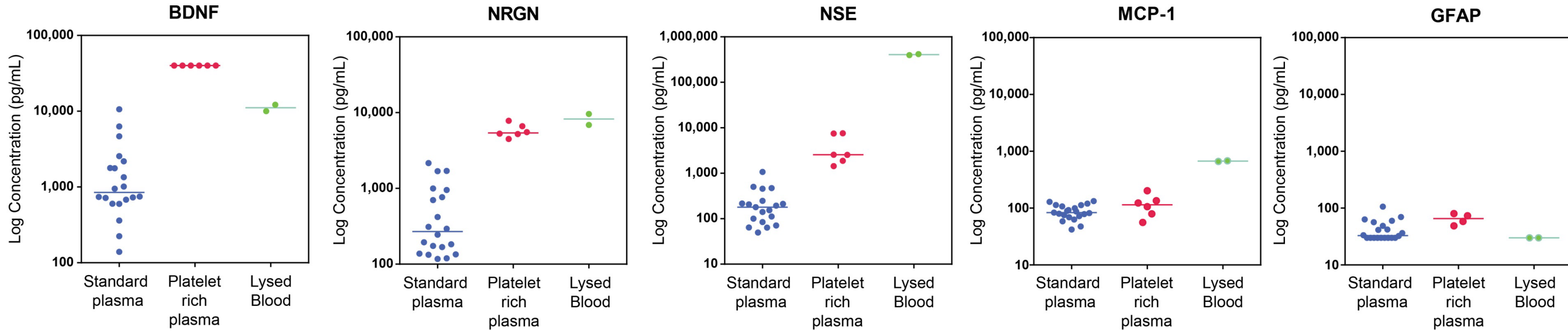
| | | GFAP | CKBB | VILIP-1 | Tau | UCH-L1 | MCP-1 | BDNF | MMP-9 | NSE | NRGN | S100B | vWF |
|--------------------|----------|------|------|---------|------|--------|-------|------|-------|-----|------|-------|------|
| Dilution Linearity | Sample 1 | 119% | 68% | 136% | 86% | 81% | 96% | 111% | 112% | 91% | 77% | 98% | 97% |
| | Sample 2 | 136% | 82% | 133% | 94% | 78% | 92% | 113% | 132% | 92% | 87% | 113% | 102% |
| | Sample 3 | 124% | 83% | 145% | 98% | 86% | 94% | 77% | 114% | 97% | 83% | 104% | 88% |
| | Sample 4 | 89% | 103% | 120% | 96% | 84% | 91% | 70% | 125% | 99% | 89% | - | 90% |
| | Average | 117% | 84% | 134% | 93% | 82% | 93% | 93% | 121% | 95% | 84% | 105% | 94% |
| Spike Recovery | Sample 1 | 82% | 108% | 50% | 108% | 136% | 94% | 107% | 87% | 71% | 120% | 92% | 104% |
| | Sample 2 | 82% | 101% | 53% | 95% | 126% | 78% | 114% | 78% | 80% | 100% | 89% | 106% |
| | Sample 3 | 64% | 98% | 59% | 97% | 138% | 80% | 100% | 85% | 76% | 110% | 94% | 105% |
| | Sample 4 | 115% | 84% | 65% | 93% | 124% | 88% | 114% | 76% | 80% | 123% | - | 90% |
| | Average | 86% | 98% | 56% | 96% | 131% | 85% | 109% | 82% | 77% | 113% | 92% | 101% |

5 Pre-Analytical Factors

Plasma levels of four of the TBI biomarkers were sensitive to variations in sample collection and processing. The apparent cause was release of biomarkers from blood cells that contained significant amounts of the markers. Lysis or disruption of blood cells (with concurrent release of biomarkers) can occur during blood collection or during subsequent processing and/or freezing. This can be exacerbated by insufficient centrifugation of the blood, particularly if the biomarkers are present in platelets or white blood cells.

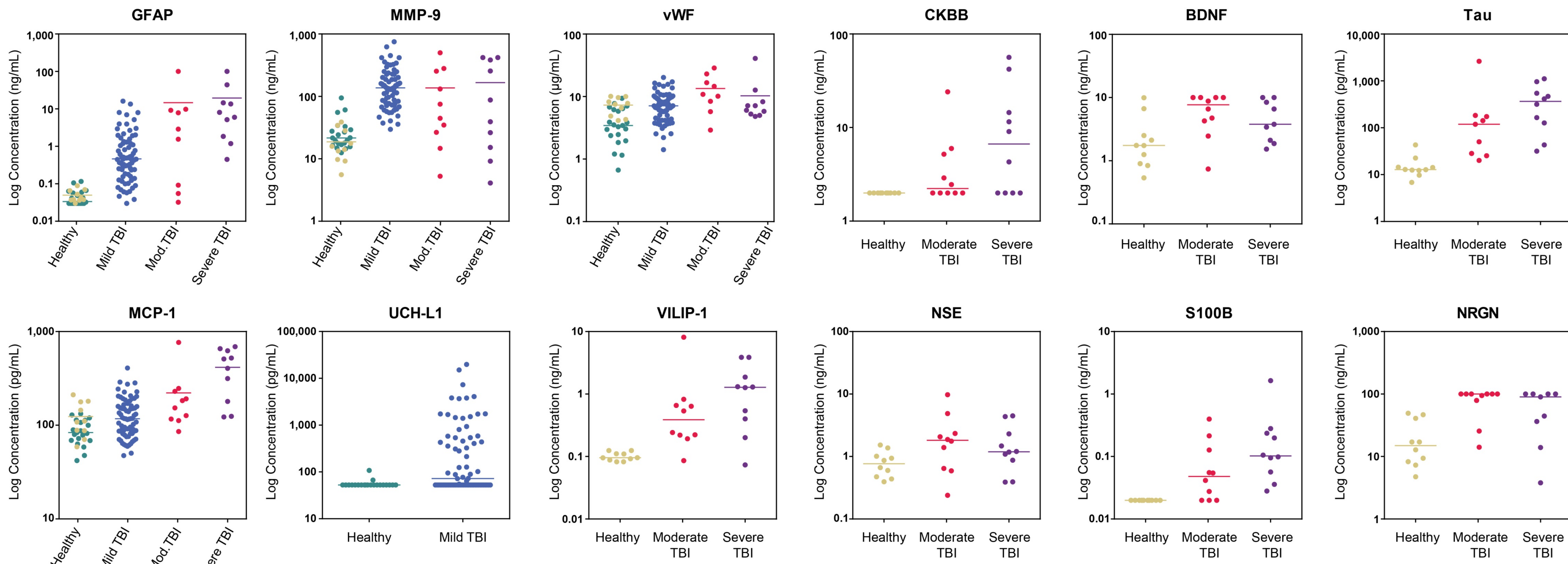
Pre-analytical effects were demonstrated by testing plasma prepared using standard centrifuge conditions (≥ 2,000 g for 20 minutes; “Standard plasma”), plasma prepared using a low centrifuge speed (200 g for 15 minutes; “Platelet-rich plasma”), and whole blood samples lysed via a freeze-thaw cycle. All samples were from healthy donors. GFAP was not affected by pre-analytical factors and is shown for reference.

For biomarker assays exhibiting pre-analytical effects (BDNF, NRGN, NSE, and MCP-1), it is recommended to avoid hemolyzed samples. Prepare plasma using standard centrifuge conditions (≥ 2,000 g for ≥ 20 minutes) and re-spin the plasma to generate platelet-poor plasma (3,700 g for 10 minutes, take top 2/3 fraction). Collection and processing of healthy control samples along with the TBI samples is also recommended to provide a baseline to help ‘subtract out’ any pre-analytical effects.



6 TBI Plasma Testing

Representative data from TBI plasma samples are shown below. Samples with undetectable biomarker levels are plotted at the assay LOD. **Moderate and Severe TBI** -- Data for moderate and severe TBI samples are shown along with healthy control samples (tan circles) collected under the same protocol. **Mild TBI** -- Since the mild TBI samples contained substantial blood cell contamination, data are shown only for those biomarkers that did not exhibit pre-analytical effects. Healthy controls for this set were not collected during the study; however, data for healthy controls collected by a vendor (green circles) or during the moderate/severe study (tan circles) using standard processing methods are shown.



7 Conclusion

- Prototype assay panels for 12 TBI-related biomarkers were developed and characterized on MSD’s MULTI-ARRAY platform.
- The high sensitivity, dynamic range, and multiplexing of MSD assays allowed all 12 biomarkers to be measured (in duplicate) in 200 µL of plasma. These assays will be used for screening large TBI sample sets under an on-going DoD funded study.
- Artificially elevated levels of BDNF, NRGN, NSE, and MCP-1 were observed in plasma due to the presence of residual blood cells that contained substantial amounts of these markers. Careful control over sample collection and processing can minimize these effects. When collecting TBI samples, inclusion of identically-processed healthy controls is recommended to help control for any pre-analytical effects.

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