Previously unrecognized, interesting and useful properties of certain antibodies to neurofilament NF-L

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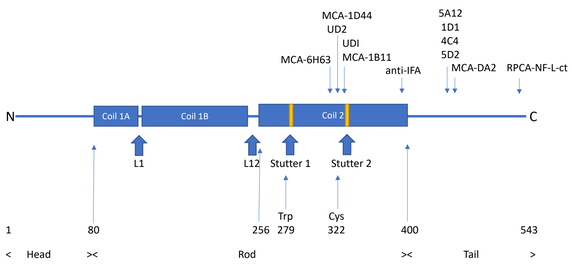
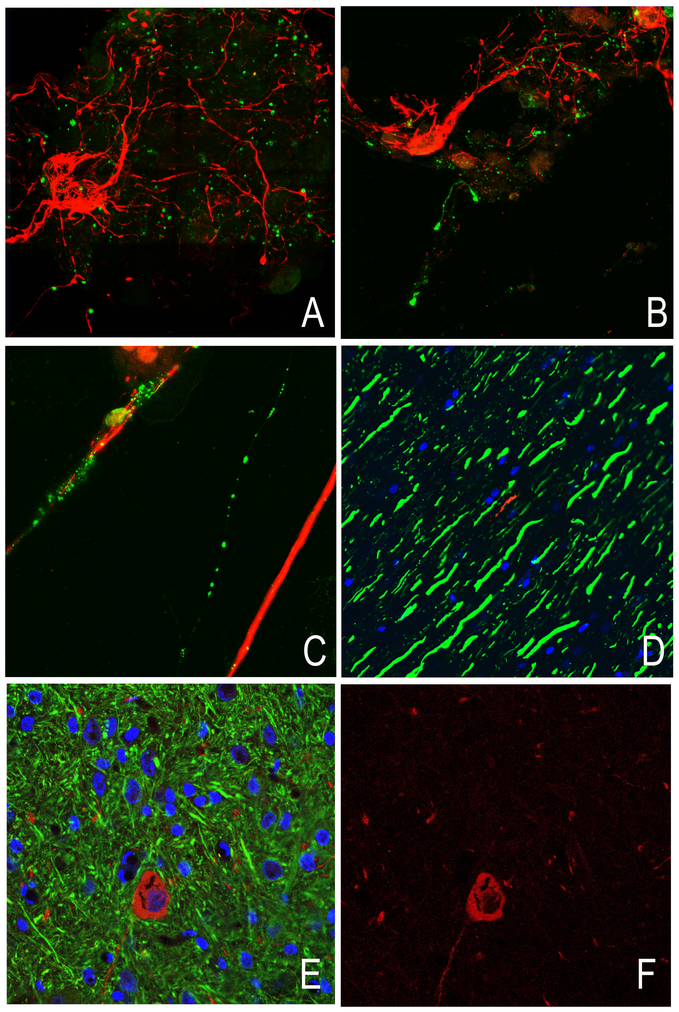
Abstract

Neurofilaments (NF) are major structures of the nervous system where they are found specifically localized in neurons and heavily concentrated in axons. Axons are peculiarly sensitive to damage and disease states. Accordingly, much interest has focused on the detection of NF subunit proteins released into blood, CSF and other biological fluids as surrogate markers of neuronal injury and degeneration. Recent work shows that the NF light chain, NF-L, can be detected at informative levels in blood and CSF after CNS injury or disease states. Much of this work has been performed using two mouse monoclonal antibodies to NF-L, UD1 and UD2, also known as 2.1 and 47.3 respectively. The antibodies are essential components of the Uman Diagnostics NF-Light™ ELISA kit as well as the Quanterix Simoa™ and other assays. Here we provide a detailed characterization of the epitopes for both reagents. We also disclose a surprising and potentially very useful feature of the Uman and similar reagents. Specifically, the Uman reagents do not recognize typical neurofilamentous profiles in healthy neurons and their processes as visualized in sectioned material or in cells growing in culture. However epitopes for both Uman reagents become accessible in degenerating neuronal cells and processes. We found that antibodies to the C-terminus of NF-L are lost during degeneration, so that healthy neurons and their processes are NF-L-ct positive and Uman antibody negative. Following degeneration, processes and aggregates become NF-L-ct negative and Uman antibody positive. These specific NF-L antibodies can therefore be used to positively identify both healthy and degenerated processes. We provide evidence that the transition from NF-L-ct positive/Uman antibody negative to NF-L-ct negative/Uman positive can be mimicked by treatment with proteases. Finally we have developed a panel of novel antibodies which also only bind degenerated processes.

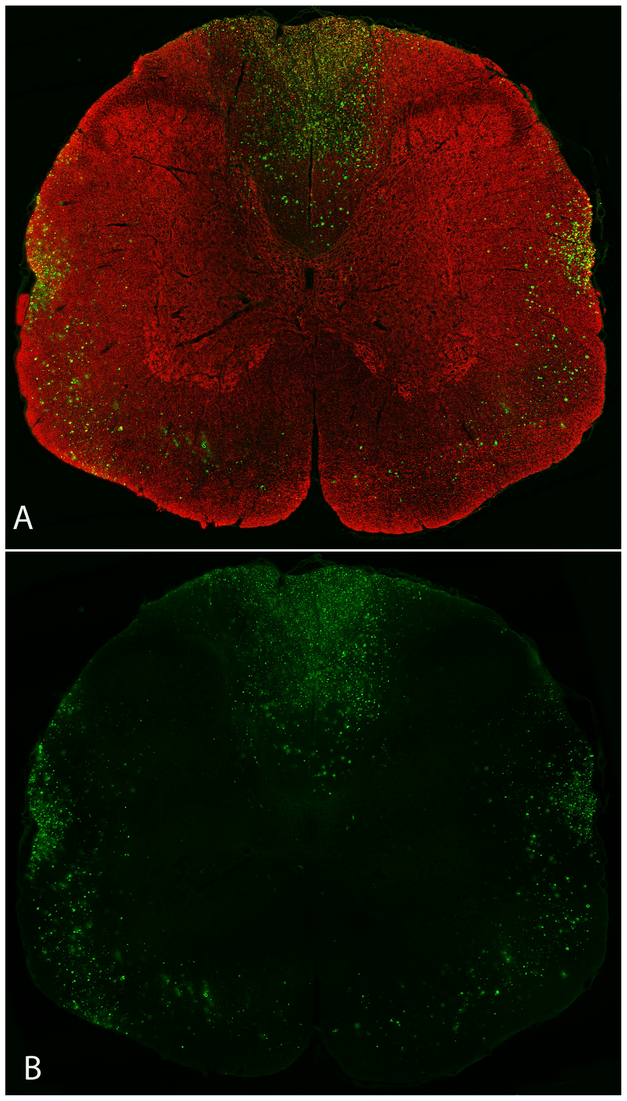
Results



Summary of Epitope Data: Diagram of the location of Uman epitopes on NF-L with relation to Trp279, Cys322 and the stutter 2 sequence. Chemical cleavage at Trp279 and Cys322 were used to localize the two Uman epitopes to a short peptide flanking the stutter 2 sequence. Using this information we epitope mapped existing antibodies and made our own panel of novel NF-L antibodies which share the unusual degeneration related staining pattern we saw with the Uman reagents. We also mapped several antibodies to the C-terminal tail of NF-L. As shown here, the epitopes for these antibodies are destroyed or removed during degeneration, providing complementary staining to that of the Uman reagents.



Above: Preliminary Immunostaining with UD1 and UD2; Panel A and B: 7-10 day neural cultures from E20 rats stained with UD1 and UD2 respectively in green, both co-stained with RPCA-NF-L-ct in red. The Uman reagents stain a few linear profiles but mostly recognize punctate NF-L-ct negative aggregates. **Panel C:** shows a region of a similar culture stained with UD2 in green and RPCA-NF-L-ct in red. The prominent linear array of Uman positive material is suggestive of the remains of a degenerated process. **Panel D:** shows a section of normal adult rat spinal cord stained with UD2 in red and RPCA-NF-L-ct in green. A single fiber is revealed with the UD2 antibody against the extensive background of "normal" NF-L. **Panel E and F:** shows a region of brain stem from a control rat. One apparently unhealthy looking neuron and associated processes is positive for UD2 but not RPCA-NF-L-ct. **Panel F:** shows only staining with the Uman antibody, note diffuse staining pattern.



Above : Coronal section of contused rat spinal cord. We damaged spinal cords of adult rats by either a C4 contusion or dorsal cut. Sections taken 1, 3 and 5 days later showed numerous Uman positive fibers against a background of RPCA-NF-L-ct processes which were mostly negative for the Uman reagents. Both Uman reagents stained identically, **Panel A:** shows a cross section of spinal cord from a rat given a cut lesion 3 days previously and stained with RPCA-NF-L-ct in red and UD2 in green. The UD2 antibody stains material in the dorsal columns, dorsal corticospinal and rubrospinal tracts, expected to be damaged by a lesion of this kind**. Panel B:** shows the UD2 signal extracted from the above specimen.

Conclusions

The data presented here contains one very surprising finding, that both Uman antibodies did not stain cells and tissues like other typical well characterized NF-L antibodies. With a few rare exceptions they did not bind neurofilaments in healthy tissues which were strongly positive with typical antibodies to NF-l and other neurofilament proteins. Clearly they are highly specific for NF-L as evidenced by their convincing binding to native mammalian and recombinant NF-L on western blots, in ELISA type assays and by definitive binding to NF-L derived peptides. We propose that the lack of staining of normal healthy cells and tissues is because the relevant Uman antibody epitopes are normally buried in assembled neurofilaments. On degeneration the Uman and related epitopes become exposed and available for antibody binding. Antibodies binding the C-terminal of NF-L generally do not stain this degenerating material, though some profiles are positive for both a Uman antibody and NF-L-ct antibody. When we induced neurodegeneration by experimental spinal cord injury we noticed a spectacular change in the Uman staining pattern with very large numbers of processes now visible. Furthermore many of these profiles were beaded, sinusoidal or discontinuous in appearance, the hallmarks of degeneration.

The unmasking of the Uman epitopes is likely due to degeneration induced proteolysis and can be mimicked by treating sections of healthy tissue with proteinase K or trypsin, resulting in previously unreactive NF-L containing profiles becoming strongly reactive with Uman type antibodies. In addition tissues which are left at room temperature for extended periods prior to fixation develop an increasing amount of Uman positive material.

We have now generated a novel panel of monoclonal and polyclonal antibody reagents directed against the same segment of NF-L as the Uman reagents which display the same interesting properties. This work shows that the region to which the Uman reagents bind includes other hidden epitopes distinct from those of either Uman reagent. The original Uman reagents and our panel of novel antibodies are robust, novel and specific markers of neurodegeneration of wide utility in future studies of CNS disease and injury.

Acknowledgements

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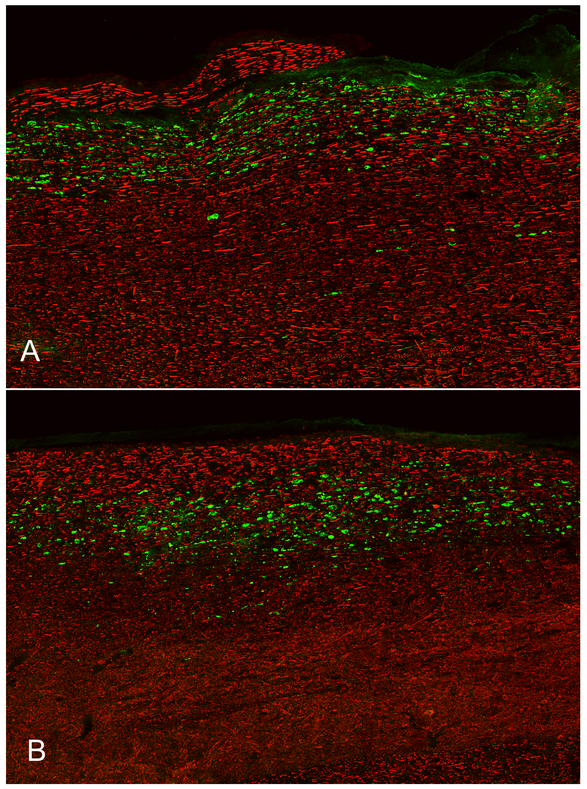
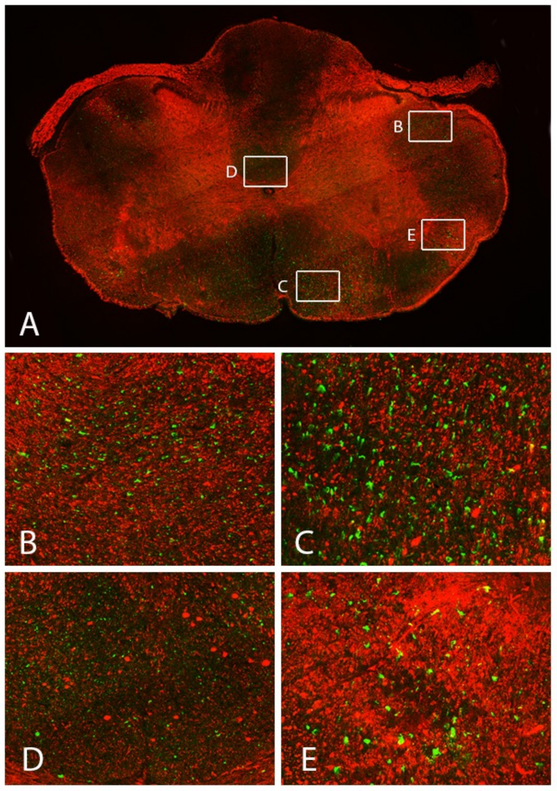
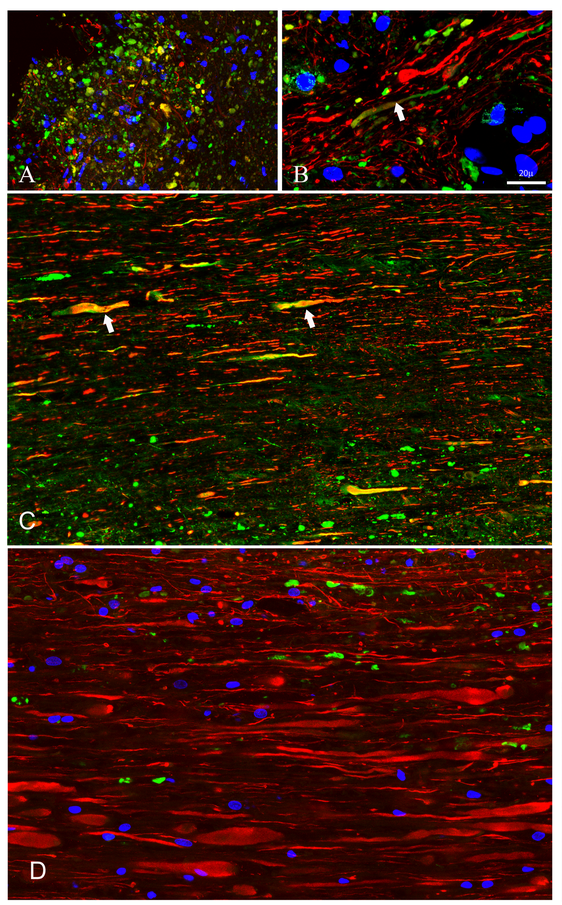


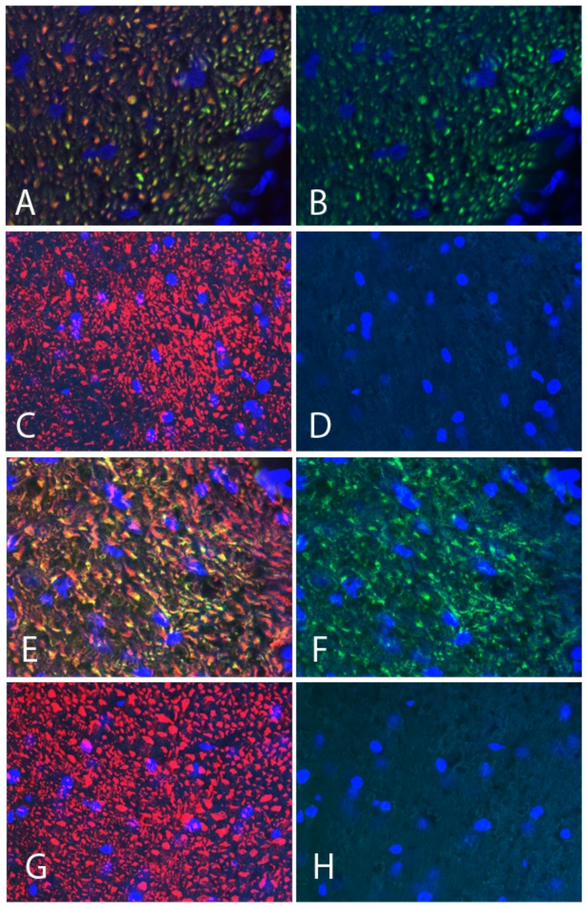
Above: Sections of spinal cord of an adult rat injured by a C4 contusion 3 days previously. Panel A: was stained with UD1 in green and RPCA-NF-L-ct in red. Numerous Uman positive fibers are seen against a background of RPCA-NF-L-ct processes which were mostly negative for the Uman reagents. **Panel B:** shows a similar longitudinal section of spinal cord stained for UD2 in green and RPCA-NF-L-ct in red. The Uman positive profiles have the properties of degenerated fibers and do not stain with NF-L C terminal antibodies.



Above: Novel monoclonal antibody MCA-6H63 raised against NF-L on injured rat spinal cord. Panel A: is low power overview of spinal cord of rat given a C4 contusion 3 days previously. Red shows staining with RPCA-NF-L-ct and green shows MCA-6H63. The staining pattern of MCA-6H63 shows almost no overlap with the NF-L-ct antibody. The MCA-6H63 pattern is also is indistinguishable from that seen with UD1 and UD2 although the MCA-6H63 antibody has an epitope distinct from either of these antibodies**. Panels B to E:** show higher magnification views of region of the section.



Transitions from NF-L-ct positive/Uman negative to NF-L-ct-negative/Uman Positive. Panel A: A region close to the lesion site in a rat given a C4 contusion 3 days previously shows RPCA-NF-L-ct in red and UD1 in green. Some profiles, presumably in the process of degenerating, are seen in yellow. **Panel B:** A process (arrowed) apparently transitioning from NF-L-ct positive/Uman negative to NF-L-ct-negative/Uman positive. **Panel C:** Other examples of apparently transitioning neuronal processes. **Panel D:** a region further away from the lesion site shows swollen but apparently still healthy processes.



Uman epitopes are revealed by protease treatment. Coronal sections of uninjured control rat spinal cords were treated with trypsin or proteinase K or with buffer control. **Panel A:** shows a region of lateral funiculus after 10 minutes in trypsin stained with RPCA-NF-L-ct in red and UD1 in green. **Panel B:** shows only the green signal. There is considerable overlap of the two antibodies. **Panel C and D:** shows a control section of a similar region incubated as the section in A and B with the same two antibodies at the same concentrations and confocal settings but without trypsin. There is a stronger RPCA-NF-L-ct signal and as shown in D, no significant UD1 signal. **Panels D and E:** show similar data for UD2 (green) and RPCA-NF-L-ct (red) on a similar trypsin treated section, and **Panels G and H:** show the staining of the same two antibodies on a control section.