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Ultrasensitive assay technology and fluid biomarkers for the evaluation of peripheral nerve disease

Authors and affiliations

Roberto Bellanti[1], Stephen Keddie[2], Michael P Lunn[3.4], Simon Rinaldi[1]

1. Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, UK
2. Department of Neuromuscular Diseases, The Royal London Hospital, Barts Health NHS Trust, London, UK
3. Department of Neuromuscular Diseases, University College London, London, UK
4. Department of Neuroinflammation, National Hospital of Neurology and Neurosurgery, UCL Institute of Neurology, London UK

Corresponding author

Associate Professor Simon Rinaldi
Nuffield Department of Clinical Neurosciences
University of Oxford
Oxford, UK
Phone: +44 (0)1865 231912
Email: simon.rinaldi@nhs.net

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Abstract

The field of biomarker discovery is rapidly expanding. The introduction of ultrasensitive immunoassays and the growing precision of genetic technologies are poised to revolutionise the assessment and monitoring of many diseases. Given the difficulties in imaging and tissue diagnosis, there is mounting interest in serum and cerebrospinal fluid biomarkers of peripheral neuropathy. Realised and potential fluid biomarkers of peripheral nerve disease include neuronal biomarkers of axonal degeneration, glial biomarkers for peripheral demyelinating disorders, immuno-pathogenic biomarkers (such as the presence and titre of antibodies or the levels of cytokines), and genetic biomarkers. Several are already starting to inform clinical practice, whereas others remain under evaluation as potential indicators of disease activity and treatment response. As more biomarkers become available for clinical use, it has become increasingly difficult for clinicians and researchers to keep up-to-date with the most recent discovery and interpretation. In this review, we aim to inform practicing neurologists, neuroscientists and other clinicians about recent advances in fluid biomarker technology, with a focus on single molecule arrays (Simoa), chemiluminescent enzyme immunoassays (CLEIA), electrochemiluminescence (ECL), proximity extension assays (PEA), and microfluidic technology. We discuss established and emerging fluid biomarkers of peripheral neuropathy, their clinical applications, limitations, and potential future developments.

Introduction

Biomarkers have the potential to revolutionise clinical practice and research. They can serve as indicators of normal or pathological biological processes, or dynamic responses to therapeutic intervention. (1)Clinically, biomarkers can aid diagnosis, monitoring of disease activity and titration of treatment to clinical response. On a research level, they may inform patient selection for observational and experimental studies, or serve as outcome measures as surrogate, secondary or even primary endpoints in trials.

Compared to biomarkers for central nervous system disease, the biomarkers of peripheral neuropathy currently available are suboptimal. Nerve specific fluid biomarkers for clinical use have not been developed, and neuropathies are currently diagnosed and monitored through neurological examination and a combination of clinical assessment, outcome scores and neurophysiology, with a limited but increasing role for peripheral nerve imaging. The traditional methods are all semi-quantitative, correlate imprecisely with underlying pathology, cannot distinguish residual damage from active disease, and are poorly responsive. Specific fluid biomarkers may simplify diagnosis, prognostication and monitoring of disease activity. Moreover, as demand, consumption and cost of intravenous immunoglobulin (IVIg) in the

inflammatory neuropathies continue to increase, responsive and reliable biomarkers are urgently needed to individually tailor all therapies and improve their cost-effectiveness.

The field of fluid biomarker discovery is rapidly expanding thanks to modern laboratory techniques with levels of sensitivity several orders of magnitude above traditional ELISA, western blotting and other technologies. Highly sensitive assays are now available on a number of platforms for the measurement of analytes at ultra-low concentrations, allowing detection of novel biomarkers across a wide spectrum of diseases. These technologies include single molecule arrays (Simoa), chemiluminescent enzyme immunoassays (CLEIA), electrochemiluminescence (ECL), proximity extension assays (PEA), and microfluidic technology. In this review, we discuss established and emerging assay technologies and fluid biomarkers for the evaluation of peripheral nerve disease, their clinical applications, limitations, and potential future developments.

Ultrasensitive immunoassays for the measurement of neuropathy fluid biomarkers

For a long time, enzyme-linked immunosorbent assay (ELISA) has been used as the practical gold standard for the measurement of fluid biomarkers. However, a major limitation of this technique is the relatively large sample volumes (50–100 µL) per test and millions of analyte molecules needed to generate detectable signal, which limits assay sensitivity. The lowest limit of detection is rarely below nanograms/millilitre (ng/ml) range, and the multitude of proteins present in blood or cerebrospinal fluid (CSF) at lower concentrations cannot be detected. Ultrasensitive and multiplexing technologies are now available to measure disease biomarkers and other molecules. These are broadly based on the same fundamental mechanism underlying sandwich ELISA: the formation of immune-complexes of antibodies binding to the analyte(s) of interest. However, novel antigen capture and display, amplification, imaging and statistical signal handling methods increase sensitivity by several orders of magnitude. These highly sensitive assays may require smaller sample volumes and can often be multiplexed, enabling the quantification of a wide range of previously unmeasurable biomarkers singly or together.

In this review, we focus on Simoa, chemiluminescence, electrochemiluminescence, proximity extension assays and microfluidic immunoassays because they are the only ultrasensitive technologies which have been used in the field of peripheral neuropathy to date. Only some of the biomarkers discussed in this review can be measured using such technologies, whereas the others still rely on traditional, less sensitive methods. All biomarkers and existing assays for their measurement are summarised in tables 1 and 2.

- **Single Molecule Arrays (Simoa)**

The introduction of single molecule array technology has had an unprecedented impact on biomarker discovery, and what would seem unachievable until a decade ago has now become routine. The ultrahigh sensitivity of Simoa can be compared to being able to detect and quantitate a spoonful of sugar dissolved in a full-size Olympic swimming pool. Compared to conventional ELISA, Simoa only requires small amounts of protein (down to 50 fg) to measure ultralow levels of proteins and nucleic acids, increasing sensitivity by many orders of magnitude, and decreasing the lower limit of detection down to the attomolar range (10^{-16} M).

Simoa relies on capture and then enzyme labelled antibody detection of target analytes on paramagnetic beads, with individual microbeads distributed in arrays of microwells for digital or analogue analyte quantification. Paramagnetic beads coupled with capture antibodies selectively bind to the analyte of choice, which is then detected by biotinylated antibodies to form an immune-sandwich. An enzyme conjugate (streptavadin β galactosidase, SBG) is used to label the immunocomplex consisting of beads, capture antibodies, analyte and detection antibodies [Figure 1], which is then re-suspended in a resorufin β-D-galactopyranoside (RGP) substrate and loaded into arrays in a disc for analyte quantification. The disc contains over 200,000 wells, each one designed to hold one bead only. A vacuum pulls the beads into each well and oil is spread over the surface of the array, removing excess beads from the array surface and sealing loaded wells. The fluorescent signal resulting from the enzymatic reaction can be generated by a single molecule of the analyte. This is captured and imaged by a camera, and at low concentrations the instrument determines the number of wells which have a fluorescent bead in an ‘on-off’ binary fashion. At higher analyte concentrations, several immunocomplexes can form in each well, producing greater signal and allowing wider dynamic ranges compared with conventional immunoassays.

- **Chemiluminescence and electrochemiluminescence**

Luminescence is the emission of light as a result of a chemical (chemiluminescence) or electrochemical (electrochemiluminescence) reaction. Chemiluminescent enzyme immunoassays and ECL platforms have been employed for the measurement of biomarkers in central and peripheral nervous system disorders.

ECL is used in combination with multiarray technology for the detection of multiple proteins in a single sample. This offers significant advantages over traditional ELISA including lower background, signal amplification, higher sensitivity and a wider dynamic range. Many platforms can also measure multiple analytes (up to 10) from a single sample at the same time (multiplexing). The majority of assays are run in multi-spot

microplates which have electrodes at the base of each well. Each spot is coated with unique capture antibodies and, following incubation with the sample, detection antibodies are added. A ruthenium SULFO-TAG is then used along with tripropylamine to catalyse a reaction that generates light when electrical current is applied to the plate electrodes, and the intensity of the emitted light is measured for analyte quantification [Figure 2]. The SULFO-TAG can directly label the detection antibody, or a secondary antibody binding to the detectors, or can be coupled with streptavidin and tag-biotinylated detector antibodies [Figure 3].

A study compared conventional ELISA, ECL and Simoa for the measurement of neurofilament light (NfL) in serum and CSF, and found that Simoa and ECL are substantially more sensitive than ELISA (detection limits were 0.62 pg/mL for Simoa, 15.6 pg/mL for ECL, and 78.0 pg/mL ELISA). (2) Limit of detection (LOD) of the latest Simoa NfL assay kits is 0.085 pg/mL, and more recent analyses have shown that lower limit of quantification (LLOQ) of ECL assays for serum NfL is as low as 3.4 pg/mL. (3)

CLEIA technology is similar to ECL in that both methods use a chemical reaction with emission of light to measure analytes. However, ECL requires an electrode surface while chemiluminescence does not. Chemiluminescent assays can multiplex, and some of them have the additional advantage of automatization which reduces intra- and inter-assay variability and increases precision of results. CLEIA has been used to measure total tau in the CSF of patients with acute and chronic inflammatory neuropathies (4), with a previously reported detection limit of 141 pg/ml. (5)

- **Proximity Extension Assays (Immuno-PCR)**

Proximity Extension Assay technology is based on the principle of immuno-polymerase chain reaction (PCR). It combines the ELISA mechanism with PCR amplification and is often referred to as “immuno-PCR”. The assay relies on capture and detection antibodies binding a target analyte and forming an immunocomplex, where the detection antibody is conjugated to an oligonucleotide sequence which is subsequently detected by a complementary oligonucleotide primer. This allows for DNA polymerase-dependent extension and PCR amplification, the signal from the immunocomplex is magnified, and the resulting assay sensitivity is 1000 higher than traditional ELISA. This form of immuno-PCR is limited by high background signals and long turnaround times due to incubation and washing. This limitation is overcome by a more recent technology based on proximity-dependent DNA ligation, where a matched pair of capture and detection antibodies bind to adjacent epitopes on the target protein. (6–8) Each antibody is conjugated to a single-stranded DNA (ssDNA) sequence specific to the target protein, which serves as a barcode for the protein and is complementary to the other ssDNA sequence. As the complementary DNA strands hybridize to each other,

they act as a primer for DNA polymerase-dependent extension in a PCR reaction, and the intensity of the signal is proportional to the concentration of the protein. [Figure 4] The use of complementary DNA sequences reduces background signal, ensures specificity and, by eliminating the wash steps normally required in traditional immunoassays, reduces turnaround times. The immuno-PCR technology has been employed for the measurement of Transmembrane Protease Serine 5, a glial biomarker discussed later in this review.

- **Microfluidic immunoassays**

Microfluidic technology, run on the enzyme-linked ligand assay (ELLA), uses cartridge-based assays to measure analytes and, similarly to traditional and digital ELISA, relies on antigen capture and enzyme-labelled antibody detection. However, each sample is split into parallel microfluidic channels, each one coated with a different capture antibody, as opposed to traditional multiplex systems where different assays are run together. [Figure 5] As such, microfluidic technology is better described as "multi-analyte" as opposed to multiplex. This parallel-plexing method increases sensitivity, with reported LOD as low as 2.7 pg/ml (9), and reduces the risk of cross-reactivity with other antibody pairs.

Fluid biomarkers of peripheral nerve disease

1. Neuronal biomarkers of axonal degeneration

Biomarkers of peripheral axonal degeneration have a wide range of potential clinical applications, including identification of nerve disease or monitoring of disease activity and treatment response. Axonal biomarkers can also be used for early identification and quantification of axonal damage, which some studies have shown correlates with poor functional outcome in the immune-mediated neuropathy Guillain-Barré syndrome (GBS) (10), and to guide management by providing a biochemical rationale for earlier or additional treatment. In chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), axonal damage is a key determinant of clinical disability and an indicator of long-term prognosis.

- **Neurofilaments** are type IV intermediate filaments found in the cytoplasm of neurons. Together with microtubules and microfilaments, they form the majority of the cytoskeleton of central and peripheral nervous system axons. Based on their molecular weight, neurofilaments can be classified as light (NfL), medium (NfM), or heavy (NfH) chain, each of which functions differently in the cytoskeletal matrix. Plasma and serum concentrations of neurofilaments can now be accurately measured in health and disease using ultrasensitive immunoassays. In Guillain-Barré syndrome (GBS), serum NfL correlates with disease severity and clinical outcomes (11). NfL has been found to be elevated in CIDP (12), Charcot-Marie-Tooth disease (13), hereditary

transthyretin amyloidosis (14), amyloid light chain (AL) amyloidosis (15), critical illness neuro-myopathy (16), vasculitic neuropathy (17), chemotherapy-induced peripheral neuropathy (18), autoimmune nodopathies (19), and diabetic neuropathy (20). *Neurofilament heavy chain* (NfH), the heavily phosphorylated larger neurofilament species, has been evaluated in patients with amyotrophic lateral sclerosis (ALS) (21), where increased concentrations in plasma, serum and CSF have been associated with faster disease progression. NfH is not a useful biomarker of disease activity in CMT, as there is no significant difference in plasma concentrations between patients and controls, and levels do not significantly change over time (22) probably because the chronicity of damage is insufficient to exceed excretion or decay kinetics. Plasma concentrations of NfH have been shown to be only marginally increased in patients with diabetic neuropathy compared to controls (23). Neurofilament heavy chain additionally appears to be analytically less suitable as a disease biomarker (24), as its higher molecular weight can lead to “hook effects” and inconsistent plasma measurements because of neurofilament aggregation (25) (24), which does not occur with NfL (26).

- **Neuron-specific enolase** (NSE), an intracellular enzyme expressed in neurons and neuroendocrine tumours, has been traditionally used as a prognostic biomarker in hypoxic ischaemic brain injury. It is higher in patients with diabetes (type 1 and 2) and peripheral neuropathy compared to those with diabetes without neuropathy. (27) NSE has been reported to have a diagnostic sensitivity of 66.3% and a specificity of 72.5% for diabetic peripheral neuropathy (27–29). However, no data are available on the longitudinal variation of NSE levels over time. Further studies are needed to clarify its clinical utility in relation to peripheral nerve disease.

- **Peripherin** is a type III intermediate filament protein which is abundantly expressed in peripheral nerve axons, with limited spinal cord expression (largely peripheral axons) and almost none in the CNS. (30) The physiological function of this protein is unknown, but it is upregulated following peripheral nerve injury which would suggest a role in neuronal regeneration. (31) The relative specificity of peripherin to peripheral nerves coupled with its similar abundance to NfL makes peripherin a promising biomarker candidate for peripheral nerve axonal damage. (32) We recently developed a highly sensitive Simoa-based immunoassay to detect serum peripherin. (33) Unlike NfL, where levels rise with both PNS and CNS damage, our data demonstrate a significant rise of serum peripherin in patients with axonal forms of peripheral neuropathy without significantly increased levels in patients with demyelinating peripheral nerve disease, chronic axonal disease, or other forms of CNS derived neuronal damage such as dementia.

- **Total tau (T-tau)** is an established biomarker of CNS axonal injury (34,35). It is expressed in both central and peripheral axons, with highest abundance in the cortex (36), where it stabilises neuronal microtubules and contributes to axonal transport. (37) Pathological aggregation of T-tau occurs in Alzheimer's disease (AD) and progressive supranuclear palsy (38,39), and elevated serum levels have been shown in patients with AD, traumatic brain injury (TBI) (40) and hypoxic brain injury from cardiac arrest. (41) An observational, single-centre, retrospective study investigating the diagnostic and prognostic value of axonal injury biomarkers in patients with inflammatory neuropathies (4) found higher CSF T-tau levels, measured using a chemiluminescent enzyme assay, in patients with CIDP compared to healthy controls. In the same study, T-tau levels in plasma were higher in GBS, CIDP, paraproteinaemic demyelinating neuropathies and multifocal motor neuropathy (MMN) versus disease-free and healthy controls. Although plasma T-tau might potentially be used to support a diagnosis of acute or chronic inflammatory neuropathy, it remains unclear whether it can be used for monitoring or prognostic purposes, as no data are available on its correlation with widely used clinical scales such as I-RODS. Crucially, it is not known whether the source of CSF T-tau in CIDP patients is central or peripheral and, as such, the degree of peripheral nerve specificity of T-tau is yet to be determined.

2. Glial biomarkers for peripheral demyelinating disorders

Demyelination is the predominant pathological correlate of the majority of GBS cases (60-80% of patients in North America and Europe). In CIDP, macrophage-related myelin damage has been proposed to play a pivotal role in the pathogenesis, and demyelination is an important contributor to disability. Dysmyelination (the lack or aberrant development of peripheral nerve myelin) is the pathological hallmark of some genetic neuropathies. A fluid biomarker capable of identifying peripheral demyelination would aid clinical management and disease classification. However, to date, no such biomarkers have been validated for clinical use. Two glial proteins, Transmembrane Protease Serine 5 (TMPRSS5) and Glial Fibrillary Acid Protein (GFAP), and a myelin sphingolipid, sphingomyelin have been evaluated as candidate biomarkers of peripheral myelin injury.

- **Transmembrane Protease Serine 5** (TMPRSS5) has been evaluated as a Schwann cell biomarker in a study where plasma of CMT1A patients and healthy controls was profiled using immuno-PCR (42). TMPRSS5 levels were significantly higher in CMT1A compared to controls, however they did not correlate with disease scores (CMTES-R, CMTNS-R), nerve conduction velocities, or age. TMPRSS5 was not

significantly elevated in other forms of CMT such as CMT2A, CMT2E, CMT1B, or CMT1X, when compared with controls. It should however be acknowledged that TMPRSS5 is the first Schwann cell-specific plasma protein that has been found to be elevated in CMT1A patients.

- Glial Fibrillary Acid Protein (**GFAP**), an intermediate filament protein expressed in both CNS and PNS, has been shown to be significantly increased in the serum of patients with chronic axonal neuropathy (vasculitic, toxic-alcoholic, diabetic, and idiopathic) compared to CIDP, MMN and controls, correlating with reductions in sensory nerve action potential amplitudes and disease severity. (43) These findings are somewhat counterintuitive as GFAP is a glial protein and would be expected to increase with peripheral demyelination as opposed to axonal damage. In the same study, serum GFAP levels did increase, although to a lesser extent, in MMN patients compared to controls, whereas there was no difference between CIDP and MMN. Longitudinal variations of GFAP over time have not been assessed. On the basis of the currently available data, GFAP would seem to have limited scope to identify and quantify demyelination, and its performance compared to neuronal neurofilaments for the assessment of axonal degeneration is unknown.

- In a prospective multicenter cohort study (44), serum and CSF levels of **sphingomyelin** were measured in patients with CIDP, axonal and demyelinating GBS, non-demyelinating central nervous system disorders, and healthy controls. A fluorescence-based assay was used, where lipid extraction was followed by enzymatic reactions hydrolysing sphingomyelin to phosphorylcholine and ceramide, followed by hydrolysis of phosphorylcholine to obtain choline, and then the oxidation of choline with formation of hydrogen peroxide and betaine. Horseradish peroxidase was used to catalyse a reaction of hydrogen peroxide with dihydroxyphenoxazine and generate resorufin, a highly fluorescent product. Sphingomyelin concentrations were significantly higher in the CSF of patients with CIDP and demyelinating GBS compared with controls, whereas serum levels did not differ across groups. The lack of peripheral nerve specificity of sphingomyelin together with normal serum levels in patients with active disease make it a less suitable clinical biomarker for peripheral nerve disease.

3. Immuno-pathogenic biomarkers

Immuno-pathogenic biomarkers are not structural nerve components released during or after injury, but may instead indicate the presence of a specific pathological process, and include autoantibodies (in the inflammatory and paraneoplastic neuropathies) and growth factors. Contrary to axonal and glial biomarkers, which can be measured in a wide range of peripheral nerve disorders, immunopathogenic biomarkers are only relevant to restricted, defined groups of neuropathies.

3.1 Autoantibodies in the immune-mediated neuropathies

Neuropathy associated antibodies can target the glycan component of glycoproteins or glycolipids, intracellular antigens, or membrane-associated functional proteins in the peripheral nerve. Some antibodies have strong well characterised causal associations with specific immune-mediated neuropathies, and their titre may correlate with treatment response, whereas the clinical and/or pathological significance of other autoantibodies is limited or uncertain. The majority of patients with acute inflammatory demyelinating polyneuropathy (AIDP) or chronic inflammatory demyelinating polyneuropathy (CIDP) however do not have currently detectable autoantibodies.

3.1.1 Anti-ganglioside antibodies

Antiganglioside antibodies are measured on many varied and heterogeneous platforms using simple and complex antigen display parameters, antibody capture and detection, and readout. These include thin layer chromatography, ELISA, PVDF line-blots and complex microarrays, being presented alone or with cholesterol, sphingolipids or sulphatides. Assays differ hugely in results, sometimes even in their fundamental detection of one antibody activity compared to another. As a general rule, antiganglioside IgG are associated with acute and IgM with chronic immune-mediated neuropathies. The presence of antiganglioside antibodies can support a diagnosis of autoimmune neuropathy but their presence does not confirm a neuropathy and absence does not exclude it. Their presence in the serum of GBS patients may correlate with axonal/nodal pathology but currently does not impact on clinical management.

- ***Anti-GM1 antibodies.*** Elevated titres of IgM antibodies against GM1 are found in 30% to 80% of patients with multifocal motor neuropathy (MMN) which presents with pure motor weakness, predominantly in the upper limbs, and conduction block on nerve conduction studies. Up to 80% of GBS patients electrophysiologically classified as AMAN (acute motor axonal neuropathy) have IgG anti-GM1 antibodies. (45) A recent study showed that high anti-GM1 IgG and IgM antibody titres at baseline and persistently raised levels of anti-GM1 IgG correlate with poor outcome in GBS. (46) In MMN, it is unclear whether anti-GM1 antibodies are pathogenic or a disease epiphenomenon (47–51), whether higher levels are associated with more severe disease, or whether falling titres can be used to judge treatment success. (52) In mouse models, the neuropathic potential of anti-GM1 autoantibodies is regulated by the local glycolipid environment as GM1 can form complexes with other neighbouring gangliosides which can mask the epitope of some anti-GM1 antibodies. (53). This variation in fine specificity may provide a mechanistic explanation for the apparently variable detection and pathogenicity of anti-GM1 antibodies as a whole.

- ***Anti-GQ1b antibodies.*** Compared to GM1 antibodies, IgG anti-GQ1b are more specific, and can be found in up to 90% of patients with Miller-Fisher syndrome (MFS), 66% of patients with Bickerstaff brainstem encephalitis (BBE), and 25% of patients with GBS (most often those with ophthalmoplegia). (54–57) MFS and BBE present with rapid-onset ophthalmoplegia and ataxia, however patients with MFS are areflexic whereas those with BBE are often hyper-reflexic and have altered level of consciousness. On the basis of their common clinical and immunological features, MFS and BBE can be referred to as "anti-GQ1b antibody syndromes". (58) False positive results from anti-GQ1b antibody assays are uncommon. These factors make GQ1b antibodies a useful tool in strongly supporting the diagnosis of MFS or BBE, but again, their utility for prognostication, treatment titration and the measurement of ongoing disease activity is less clear.

- ***Other antiganglioside antibodies****.* Other antiganglioside antibodies target GD1a, GD1b, GD3, GT1a, and GT1b, and can be of the IgG or IgM isotypes. IgG antibodies against GD1a are found in acute motor axonal neuropathy (AMAN) and acute motor and sensory axonal neuropathy (AMSAN) which together represent 10-15% of Guillain-Barré syndrome cases. Anti-GT1a IgG antibodies often occur in patients with the pharyngeal-cervical-brachial (PCB) variant of GBS, where their presence constitutes a supportive diagnostic criterion. (59) IgM antibodies against GD3, GD1b, GT1b (and GQ1b) can be found in CANOMAD (chronic ataxic neuropathy, ophthalmoplegia, monoclonal gammopathy, cold agglutinins and anti-disialosyl antibodies), a paraproteinaemic neuropathy with IgM paraprotein. (60)

3.1.2 Paraproteins

A paraprotein (or M-protein or monoclonal gammopathy) is a monoclonal immunoglobulin secreted by a population of clonally expanded B cells. A normal immunoglobulin is formed by a heavy chain, which determines the isotype (IgM, IgG, or IgA), and a light chain (kappa or lambda). A paraprotein can be found as complete (formed by both heavy and light chain) or as a light chain only (kappa or lambda paraprotein). Paraproteins can be found in 3-4% of people over 50 years of age (61), and 10% of patients with a neuropathy of unknown cause have a paraprotein. (62) It can be difficult to establish whether a paraprotein is clinically relevant. A pathological association is more probable if the paraprotein is IgM and/or the neuropathy demyelinating. (63) More than half (50-75%) of the paraproteinaemic neuropathies are IgM versus 17-35% IgG and 8-15% IgA. (63–65) The list of monoclonal gammopathies associated with peripheral nerve disease include monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma, lymphoplasmacytic lymphoma (including Waldenström's macroglobulinaemia), non-Hodgkin's

lymphoma, chronic and small lymphocytic leukaemias, POEMS syndrome (Polyneuropathy, Organomegaly, Endocrinopathy, Monoclonal gammopathy - almost always lambda - and Skin lesions), Castleman Disease and other lymphoproliferative disorders. (63)

The differential diagnosis of a paraproteinaemic neuropathy depends on which monoclonal gammopathy is identified, IgM or IgG/IgA. Neuropathies associated with IgM paraproteins include anti-MAG neuropathy, MMN, CANOMAD, peripheral nerve neurolymphomatosis, and IgM deposition disease. Neuropathies associated with IgA or IgG paraproteins include POEMS syndrome, CIDP and amyloidosis. (63)

Although CIDP with a coincidental paraprotein is possible, current guidelines acknowledge that their presence is a “red-flag” for an alternative diagnosis, notably anti-MAG neuropathy in the presence of an IgM paraprotein, and POEMS, AL amyloid or myeloma in the presence of an IgG or IgA paraprotein. Nevertheless, the presence of a paraprotein may indicate a greater chance of response to rituximab in patients with CIDP, where pharmacological B-cell depletion can improve clinical response and decrease dependence on first-line treatments. (66,67) The presence of a paraprotein with a neuropathy does not however imply pathogenicity, or even relevance, and most are unrelated to the neurology found.

3.1.3 Anti-MAG antibodies

IgM antibodies targeting myelin associated glycoprotein, commonly referred to as anti-MAG antibodies, are found in 50% of patients with IgM paraproteinaemic demyelinating neuropathy. These antibodies are associated with a distal acquired demyelinating neuropathy which more frequently occurs in older men. Patients with anti-MAG neuropathy typically have a presentation with early unsteadiness and sensory ataxia, tremor, minimal and distal weakness, mild loss of pinprick sensation, and striking severe loss of vibration often up to the costal margins compared to minimal small fibre dysfunction. Electrophysiology shows distal, symmetrical demyelination with disproportionately prolonged distal motor latencies. Widely spaced myelin on nerve biopsy is pathognomonic.

Testing of anti-MAG antibodies should be pursued only when clinical phenotype is highly suggestive, in the presence of an IgM paraprotein, and clinicians should not precipitously infer causality when interpreting a positive result. The MAG antibody assay is a commercial ELISA where results are expressed in Bühlmann titre units (BTU) and the recommended cut-off for a ‘positive’ result is 1000 BTU. However, a recent study found a better combination of sensitivity/specificity at > 1500 BTU versus > 1000 BTU, and the highest specificity (100%) was obtained for titres > 7000 BTU. (68) Results can therefore be classified as follows: negative (<1000 BTU), weakly positive (1000-1500 BTU), positive (1500-7000 BTU), and

strongly positive (>7000). A “positive” but not “strongly positive” result lies within a grey area where antibody titre should be carefully correlated with phenotype and neurophysiology to differentiate MAG neuropathy from other neuropathies, including CIDP, to avoid misdiagnosis. When a diagnosis of MAG neuropathy is established, antibody titre should *not* be used as a monitoring biomarker as it correlates poorly with severity and response to treatment (69,70). However, IgM paraprotein levels themselves can serve as a biomarker of disease activity. One retrospective analysis recently found that a relative reduction in serum anti-MAG IgM antibodies is associated with a clinical response to immunotherapies, and a sustained reduction of at least 50% compared with pre-treatment titres may indicate therapeutic response.(71) However, no substantial evidence exists to date to support the clinical use of MAG antibodies to discriminate active versus quiescent or remitting disease.

3.1.4 Paranodal-nodal antibodies

Another group of antibodies targeting peripheral nerve antigens includes immunoglobulins of the IgG isotype (predominantly IgG4) against nodal neurofascin 186 (NF186), paranodal neurofascin 155 (NF155), contactin-1 (CNTN1) or contactin-associated protein-1 (CASPR1). These antibodies are collectively called paranodal-nodal antibodies (PNAbs). (72) The primary utility of PNAbs is to identify patients who otherwise meet diagnostic criteria for GBS or CIDP but have an immunologically and pathologically distinct disorder, more appropriately called “autoimmune nodopathy”. Such patients often have additional clinical features and respond poorly to conventional therapies (corticosteroids, IVIg, plasma exchange). Paranodal/nodal antibody-mediated diseases are considered “not-to-miss” conditions as targeted immunotherapy with the B-cell depleting monoclonal antibody rituximab may be more effective if given early in the disease course. (66,73,74)

Paranodal and nodal antibodies seem to correlate with clinical phenotype. Patients with NF186 antibodies or pan-neurofascin antibodies (which target NF155, NF140 and NF186) tend to have a severe neuropathy with subacute/acute onset, and often have associated autoimmune disorders. NF155 patients are usually younger and develop distal weakness, ataxia and tremor. Differently, CNTN1-positive patients tend to be older (although paediatric cases have been described) and develop an aggressive, motor-predominant neuropathy with early axonal loss. Nephrotic syndrome is increasingly identified as an associated condition, particularly in those with CNTN1 antibodies. (75–79) Finally, CASPR1 patients tend to have a painful neuropathy.

Recently, IgG4 **anti-LGI4 antibodies** have been found in four patients with CIDP presenting with weakness and subacute sensory impairment. One out of four had the multifocal acquired demyelinating sensory and

motor (MADSAM) variant and the others were classified as typical CIDP. CSF protein was moderately raised (1,82-5,41 g/L) and IVIg partially effective in all of them. Further studies are needed to define whether these antibodies do have a pathogenic link and are of clinical utility in the management of inflammatory neuropathies.

3.1.5 Other antibodies

Other autoantibodies which have an association with neuropathy include anti-Hu, anti-FGFR3, anti-AGO, plexin-D1 and trisulfated heparin disaccharide, and sulphatide antibodies. Although these can associate with neuropathy phenotypes, any pathogenic potential has neither been demonstrated, nor should be assumed, especially as many of the target antigens are intracellular.

3.2 Growth factors

VEGF, an angiogenic factor produced by many cell types including platelets, macrophages, tumour cells, keratinocytes, and kidney mesangial cells, is an established diagnostic and monitoring biomarker in POEMS syndrome. Plasma VEGF was initially evaluated as a discriminating biomarker of vasculitic neuropathy versus GBS, CIDP, and ALS, with lower levels following treatment with corticosteroids or other immunosuppressants (80). High serum levels of VEGF, in the presence of lambda light chain paraproteinaemia and demyelinating peripheral neuropathy, are highly sensitive and specific for POEMS syndrome. In a cohort study evaluating 195 patients, the sensitivity of raised serum VEGF for the diagnosis of POEMS syndrome was 100%, specificity was 91% in patients with peripheral neuropathy, and 92% in patients with both neuropathy and monoclonal gammopathy, and levels were significantly higher before treatment (81). Such findings suggest that VEGF, although not a structural nerve component, can be used to discriminate POEMS syndrome from other inflammatory demyelinating neuropathies.

Serum levels of **nerve growth factor** (NGF) increase in cancer patients receiving taxanes or platinum who develop painful chemotherapy-induced peripheral neuropathy (CIPN), with one paper suggesting that NGF might be a potential biomarker of neuropathy and neuropathic pain severity in this population. (82) Those findings were in contrast with a previous study, evaluating predictors of peripheral neurotoxicity in cisplatin and paclitaxel combination chemotherapy, which had shown a significant correlation of *decreasing* levels of serum nerve growth factor (NGF) with CIPN severity but not with final neurological outcome. (83) Depletion of serum levels of NGF also appear to occur in patients with haematological malignancies following treatment with bortezomib, thalidomide, or vincristine. (84)

Similarly to NGF, serum levels of **brain-derived neurotrophic factor** (BDNF) have consistently been shown to decrease in patients with

haematological malignancy receiving treatment with paclitaxel, bortezomib, or vincristine. (85–87)

Genetic biomarkers

In relation to peripheral neuropathy, genetic biomarkers include Charcot-Marie-Tooth disease (CMT) and hereditary transthyretin (hTTR) amyloidosis causative genes, microRNAs, MYD88 and CXCR4 mutations. Although genetic therapy for many neurological and neuropathic diseases is rapidly approaching, the main role of genetic diagnosis at present is to secure a molecular diagnosis and register patients with gene abnormalities for future trials if they occur. The only exception is **hTTR amyloidosis**, where gene silencing therapy with antisense oligonucleotides or small interfering RNAs are licensed in patients with evidence of peripheral nerve involvement.

Over 100 **CMT causative genes** have been identified so far, however more than 60% of patients have one of five gene mutations: the duplication or deletion of the 17p chromosome or mutations of PMP22, GJB1, MPZ and MFN2. (88–90) The technological progress and the expansion of genetic panels, which often contain over 100 genes, have increased the number of variants of unknown significance (VUS) and overall rendered genetic counselling more challenging. For this reason, a careful clinical assessment is essential to phenotype the neuropathy and assess the patient as well as unaffected family members.

Studies have investigated whether **MicroRNAs** (miRNAs) can identify and diagnose inherited neuropathies. They are small non-coding RNAs (18–22 nucleotides) that regulate gene expression at post-transcriptional level and are involved in a wide range of biological processes and, although not validated for clinical use, have been evaluated as potential biomarkers across a broad spectrum of diseases. A study evaluating whether microRNAs are significantly elevated in the plasma of individuals with CMT1A compared to healthy controls identified a set of micro-RNAs specific to Schwann cells and muscle that are elevated in two separate CMT1A patient cohorts. (91) A previous study had shown that high plasma levels of miR-181, a miRNA enriched in brain and spinal cord neurons, predict disease course and a two-fold increased risk of death in ALS. (92) However, the evidence for their use in clinical practice to classify, stratify or monitor genetic or other peripheral neuropathies is currently scarce, and further studies are needed to evaluate their utility.

MYD88 L265P is a somatic point mutation of the myeloid differentiation factor 88 gene which causes an amino acid change from leucine to proline. MYD88 is associated with a number of underlying lymphoproliferative disorders, most often (>90% of cases) Waldenström macroglobulinaemia (WM). The same mutation is present in 60% of MAG neuropathy patients, according to a recent study where analysis was

performed on bone marrow aspirates or trephine (93), and where it may suggest a pathogenetic link with WM, and about half of IgM MGUS (93), whereas it is absent in multiple myeloma and non-IgM MGUS. MYD88 mutation analysis can be clinically helpful when alternative diagnostic measures (including bone marrow biopsy) have been inconclusive. The mutation only occurs in the clonally expanded cells but its presence does not necessarily imply a haematological malignancy. As such, MYD88 mutated cells have the capacity to become neoplastic but are not necessarily so. MYD88 positive WM patients are more likely to respond to ibrutinib, an oral Bruton's tyrosine kinase inhibitor compared with wild-type MYD88 patients. (93)

After MYD88 L265P, **CXCR4 i**s the second most common genetic mutation in WM, where it can be found in 24-50% of cases. (94–96) CXCR4 mutation has also been reported in 4–20% of IgM MGUS patients. (97) Therefore, similarly to MYD88, if found in a patient with peripheral neuropathy, CXCR4 mutation most probably indicates underlying WM or IgM MGUS. The same mutation causes another genetic condition called WHIM (Warts, Hypogammaglobulinemia, Infection and Myelokathexis) syndrome, where patients have recurrent infections due to white cell sequestration in the bone marrow (myelokathexis). The difference between this condition and WM is that in the former the mutation is ubiquitous, whereas in WM it can only be found in the malignant cells. For this reason, CXCR4 is also referred to as "WHIM-like" mutation, however patients with WHIM syndrome are not known to develop peripheral neuropathy.

Future directions

Future biomarker research must identify serum biomarkers that are specific to the peripheral nervous system and highly sensitive to underlying pathological changes. This is particularly important in chronic inflammatory neuropathies where excessive immunosuppression can lead to unnecessary side effects, and treatment failure is associated with impact on long-term outcome. Specific nerve biomarkers will also allow identification and quantitation of peripheral nerve involvement in conditions with additional systemic or CNS involvement such as hTTR amyloidosis.

Conclusion

There is mounting interest in serum and CSF biomarkers of peripheral neuropathy. Ultrasensitive immunoassays are increasingly used to measure fluid biomarkers of nerve injury and, along with the increasing precision of genetic technologies, show great promise. New fluid biomarkers may aid clinical management, improve research, and accelerate advances in the care of patients with peripheral nerve disease.

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Figure legends

Figure 1. (A) In Simoa assays, paramagnetic beads coupled with capture antibodies selectively bind to the analyte of interest. This is then detected by biotinylated antibodies to form an immune-sandwich, labelled by the enzyme (SGB) and re-suspended in a substrate (RGP) before reading and analyte quantification. **(B)** The immune complex is then transferred to a disc and wells containing the analyte will emit a fluorescent signal.

Figure 2. Electrochemiluminescence technology. When an electrical potential is applied, the ruthenium (Ru) SULFO-TAG and tripropylamine (TPA), both electrochemically active, react and emit light. The ruthenium label is oxidized at the electrode surface and, simultaneously, TPA is oxidized to a radical cation that loses a proton. The resulting TPA radical reacts with oxidized ruthenium, which shifts to an excited state and decays, emitting a photon (620 nm).

Figure 3. Schematic representation of the three possible electrochemiluminescence immune sandwich configurations. In all combinations, the capture antibody (red) selectively binds to the analyte (black), which is then detected by the detection antibody (green). **(A)** Direct tagging of the detection antibody (green); **(B)** SULFO-TAG coupled with streptavidin (violet) binding to biotinylated detector antibody (green); **(C)** tagged secondary antibody (light blue) binding to detector (green).

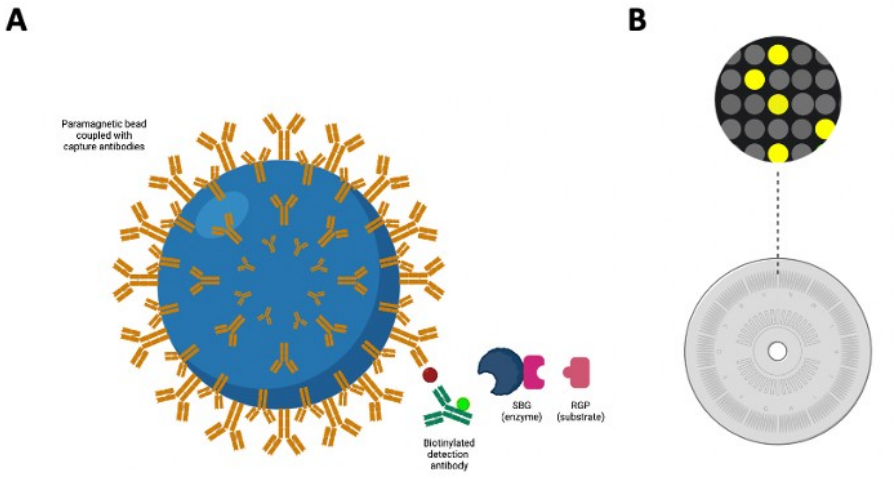
Figure 4. Proximity Extension Assay (PEA) technology. (A) A pair of antibodies labelled with DNA oligonucleotides bind their target analyte. (B) Brought into proximity, oligonucleotides hybridize and are extended by a DNA polymerase. (C) This newly formed DNA barcode is then amplified by PCR ready for readout by qPCR.

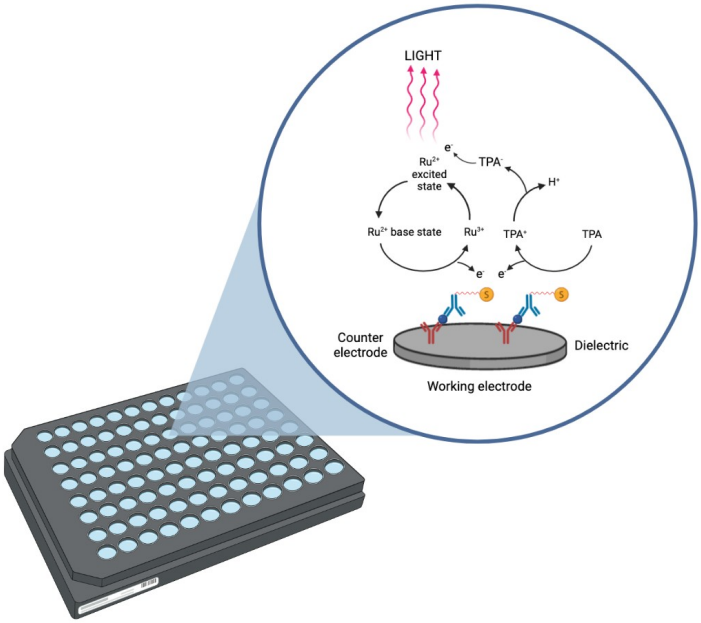
Figure 5. Schematic representation of a microfluidic immunoassay. Each sample is split across four parallel, isolated microfluidic channels. Each channel has a single-plex immunoassay for a specific analyte.

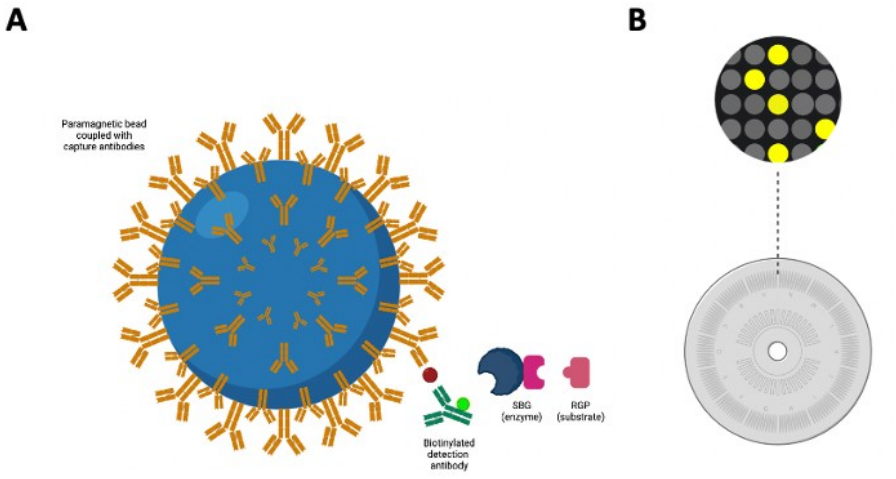
Table 1. Axonal and glial peripheral nerve biomarkers.

Table 2. Immunopathogenic biomarkers. CBA, cell-based assay; PCB, pharyngeal-cervical-brachial variant of GBS; SPE, serum protein

electrophoresis; IF, immunofixation. PN, peripheral neuropathy; CV, conduction velocity; NCS, nerve conduction studies.


A
B
Paramagnetic bead coupled with capture antibodies
SBG (enzyme)
RGP (substrate)
Biotinylated detection antibody


LIGHT
e⁻
Ru²⁺ excited state
TPA⁻
H⁺
Ru²⁺ base state
Ru³⁺
TPA⁺
TPA
e⁻
e⁻
Counter electrode
Dielectric
Working electrode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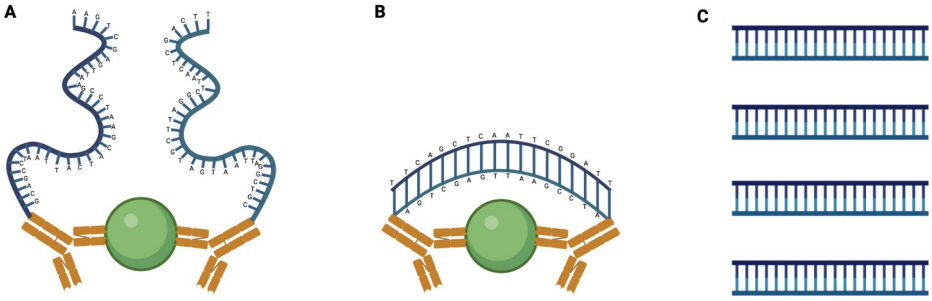
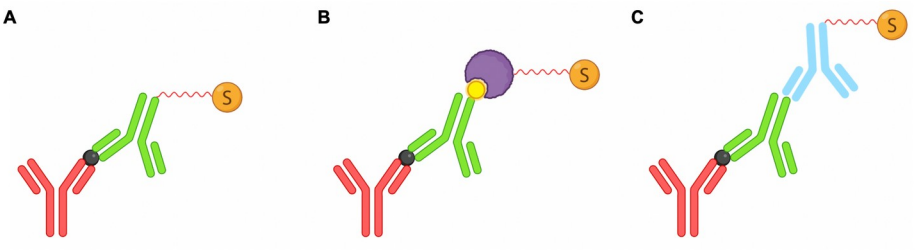
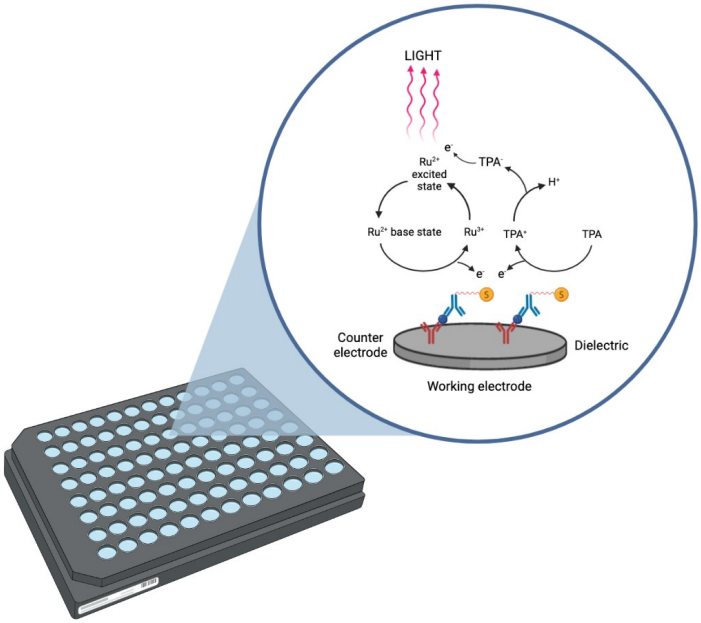
Paramagnetic bead coupled with capture antibodies
SBG (enzyme)
RGP (substrate)
Biotinylated detection antibody


A
B
C


A
S
B
S
C
S


LIGHT
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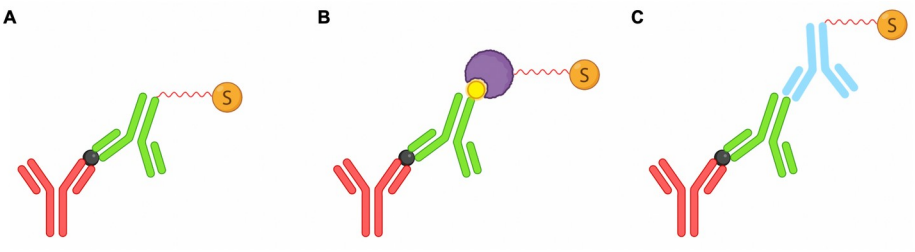
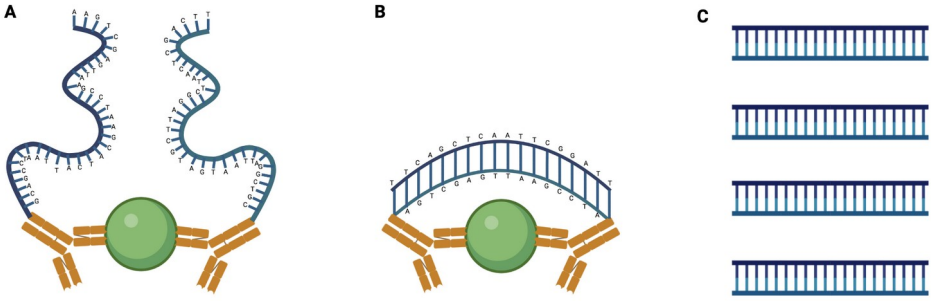
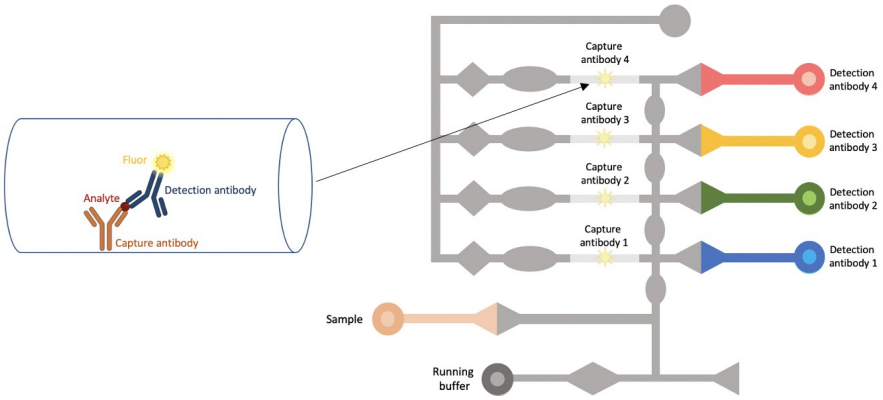

A
S
B
S
C
S


A
B
C


Fluor
Analyte
Detection antibody
Capture antibody
Capture antibody 4
Capture antibody 3
Capture antibody 2
Capture antibody 1
Detection antibody 4
Detection antibody 3
Detection antibody 2
Detection antibody 1
Sample
Running buffer