REVIEW: NEUROSCIENCE FOR NEUROLOGISTS

Nanotechnology for neuronal ion channels

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Ion channels provide the basis for the regulation of electrical excitability in the central and peripheral nervous systems. This review deals with the techniques that make the study of structure and function of single channel molecules in living cells possible. These are the patch clamp technique, which was derived from the conventional voltage clamp method and is currently being developed for automated and high throughput measurements; and fluorescence and nano-techniques, which were originally applied to non-biological surfaces and are only recently being used to study cell membranes and their proteins, especially in combination with the patch clamp technique. The characterisation of the membrane channels by techniques that resolve their morphological and physical properties and dynamics in space and time in the nano range is termed nanoscopy.

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CHANNELS—ESSENTIAL FOR NEURONAL EXCITABILITY

Ion channels are proteins that are equipped with a membrane spanning ion conducting pore. Most channels possess gates and voltage or ligand sensors. They activate—that is, open—in response to ligands, especially neurotransmitters, or voltage changes, and desensitise or inactivate—that is, close—by a normally intrinsic inactivation process. They are the molecular basis for intracellular signal transduction, maintenance of the resting potential, and the generation of excitatory and inhibitory potentials, particularly the action potential, the basic element of information in the brain.

Ion channels are integral proteins in the outer and inner membranes of excitable and non-excitable cells. Their essential property is a membrane spanning pore that acts as a conducting pathway for the flow of ions between the intracellular and extracellular spaces (fig 1). As a special feature, ion channels are opened and closed by gates. Channel opening (activation) may be effected by ligands, transmitters, a force directly acting on the channel, or changes of the transmembrane voltage. Special voltage sensing transmembrane segments of the protein control the gates in those channels that are responsible for the generation of excitatory and inhibitory subsynaptic potentials and the action potentials. They provide the basis for the regulation of excitability in the central and peripheral nervous system and the skeletal muscle (fig 2).

The structure of the channels has been elucidated by pioneer work of the McKinnon group.[1][2] Structures of importance like those determining selectivity filters, voltage sensors, ligand binding sites, and gates have been highly conserved for more than 600 million years. Thus by creating closely related channel siblings, evolution has developed on the basis of a few mechanisms a way to generate a host of physiological functions, including possibilities for compensating for functional defects.

HISTORY OF THE VOLTAGE CLAMP TECHNIQUE

Modern research into the properties of ionic channels was initiated by the pioneering work of Hodgkin and Huxley,[3] who employed the voltage clamp technique using intracellular microelectrodes to provide the first detailed description of the ionic basis of the action potential in nerve axons. Their work provided our first look at some of the functional properties of voltage gated sodium and potassium membrane *particles* before the first proof of the existence of channel proteins. For the following 50 years, the voltage clamp became the principal tool for the study of channels. Three more recent developments have revolutionised this field. The first is the patch clamp technique, developed by Neher and colleagues,[4] which is a specific application of voltage clamping developed to record the current through a membrane patch conducted by a single channel molecule. The second is the use of molecular cloning techniques to isolate channel genes, thereby determining the primary structure of the channel. The third is fluorescence microscopy, which makes functional analysis of channel ensembles and single channel molecules possible.

The patch clamp technique allows direct electrical measurement of ion channel currents while simultaneously controlling the cell's membrane potential. It relies on the use of a fine tipped glass capillary to make contact with a patch of a cell membrane in order to form a giga-ohm seal. This high resistance seal was originally applied to skeletal muscle fibres by enzymatic treatment that removed the basal membrane, glycocalix, and connective tissue. The treated preparation allowed Neher, Sakmann, and

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Abbreviations: AFM, atomic force microscopy; BNFC, benign neonatal familial convulsions; FRAP, fluorescence recovery after photobleaching; FRET, fluorescent resonance energy transfer; TIRF, total internal reflection fluorescence
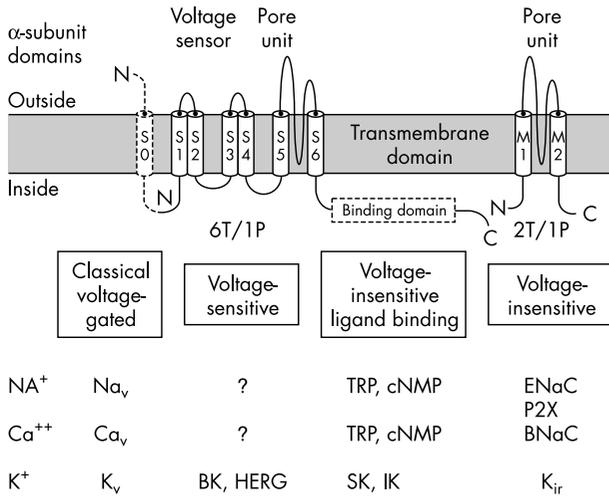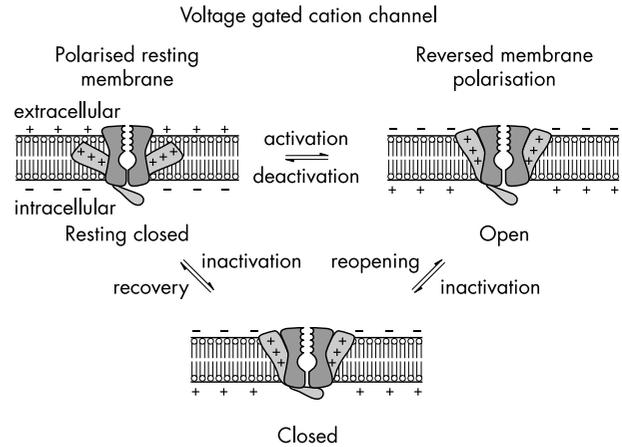
Figure 1 Diversity of domains forming cation channel α subunits. The most simple domain, typically used for ingoing rectifier potassium channel α subunits, is a pore unit (2T/1P) that consists of two transmembrane segments M1 and M2, an extracellular loop dipping into the membrane and lining the pore, and intracellular N- and C-terminals. The transmembrane segments are thought to be α helices. All voltage gated α subunit domains are 6T/1P domains as they contain a four transmembrane segment unit, S1 to S4, acting as voltage sensor, and the two transmembrane pore unit. S4 is the particular voltage sensing segment that contains positive charges at each third amino acid residue. Ligand gated cation channel α subunit domains usually possess a C-terminal binding site in addition to the 6T/1P domain. Although some ligand gated channels—for example, the calcium activated SK potassium channel—contain a positively charged S4 segment, they are not voltage sensitive at all, maybe because of uncoupling of sensor and activation gate. BK (big conductance K+) potassium channels possess an additional S0 segment. The following channels complete the classification of the α subunit domains: HERG, a potassium channel encoded by the human ether-a-go-go related gene that is similar to the *drosophila* ether-a-go-go gene (eag); IK, a calcium activated potassium channel with intermediate conductance; cNMP or CNG, sodium or calcium channels that are cyclo-nucleotide monophosphates gated such as cGMP; and voltage insensitive sodium channels of epithelial cells (ENaC) and in free nerve terminals of the brain (BNaC). BNaC was later found to conduct calcium. Not shown is another group of α subunit domains, 4T/2P, which contain four transmembrane segments and two pore units.


Figure 2 Scheme of three potential states of voltage gated cation channels such as those for sodium, calcium, and potassium ions. The resting closed channel state (upper left panel) is activated by membrane depolarisation which causes a fast transition to the open state (upper right). Owing to an intrinsic inactivation as present in sodium and calcium channels and also in some potassium channels, the channel closes (lower panel) and reopens very rarely. Repolarisation of the membrane leads to recovery from the inactivated (refractory) state back to the resting state (upper left) from which activation is again possible. There are probably more than one open and at least two inactivated (fast and slow) states (not shown). Note that transition from the resting to the inactivated state is also possible without channel opening, particularly during slow depolarisation (so called accommodation). Note also that the amphipathic voltage sensor helices move within the lipid bilayer when the membrane polarity changes. The voltage sensors are formed by the S3/S4 segments (see fig 6) and act as a "paddle" in the membrane. Modified after Jiang et al, 2003.[1] [2]

colleagues to attach the glass pipette to the plasma membrane with a leak resistance of 10 to 50 megohm. Suction resulted in a giga-ohm seal and enabled the measurements of currents in the 50 pA range with little noise.

Current variants of this technique make possible the application of solution on the exterior and interior of whole cells and on membrane patches torn from the cell (outside-out or inside-out)—every conceivable configuration of solution and ion channel orientation that the heart of ion channel researcher craves (fig 3). Usually, primary cultured cells or cell lines are preferred as they reveal a relatively clean surface membrane[5] and require no enzymatic treatment likely to damage the plasma membrane. The patch clamp technique is now the gold standard measurement for characterising and studying ion channels, and one of the most important methods applied to neurophysiology.

Single channel recordings have shown that many channels—for example, the voltage gated sodium channel—possess two conductance levels: zero when the channel is closed, and a constant conductance when the channel is open (fig 4B, 4D). Following depolarisation, there is a brief delay before channels open. The intervals are not identical during each depolarisation; in fact, the opening and closing of a given single channel is a random process, even though the

open probability depends on the voltage and is more sensitive to the voltage than an electronic device such as a transistor. After a subsequent short interval—the open time—the current jumps back to zero as the channels close.

The stochastic nature can be understood by certain energy barriers that must be overcome before a channel can flip from one conformation (for example, open) to another (closed). The energy needed for this purpose comes from the random thermal energy of the system. One can imagine that each time the channel molecule vibrates, bends, or stretches, it has a chance to surmount the energy barrier. Each motion is like a binomial trial with a certain probability of success. As the protein movements are on a picosecond time scale, but the channel stays open for milliseconds, clearly the chance of success at each trial must be small, and a large number of trials will be needed before the channel shuts.

Usually, a normal sodium channel does not reopen even though the depolarisation may be maintained by the voltage clamp step for a certain time (fig 4B). Remarkably, the average behaviour of a single channel is identical in time course to the macroscopic, whole cell sodium current (not shown).

By combining the patch clamp with molecular cloning techniques, the function and significance of potentially important amino acid residues are rapidly being elucidated. A common approach in these studies is as follows. The gene of interest is cloned and then a designed or naturally occurring mutation is inserted into the clone by mutagenesis in the hope that the mutation will produce measurable changes in channel function. Then a heterologous expression system—for example, a cell line of a different tissue which does not endogenously express the gene of interest—is used to express the gene. This expression can either be transient, as in RNA injected *Xeno*pus oocytes, or stable, as in virus infected or transfected cells (fig 4A, 4C). Finally, the patch
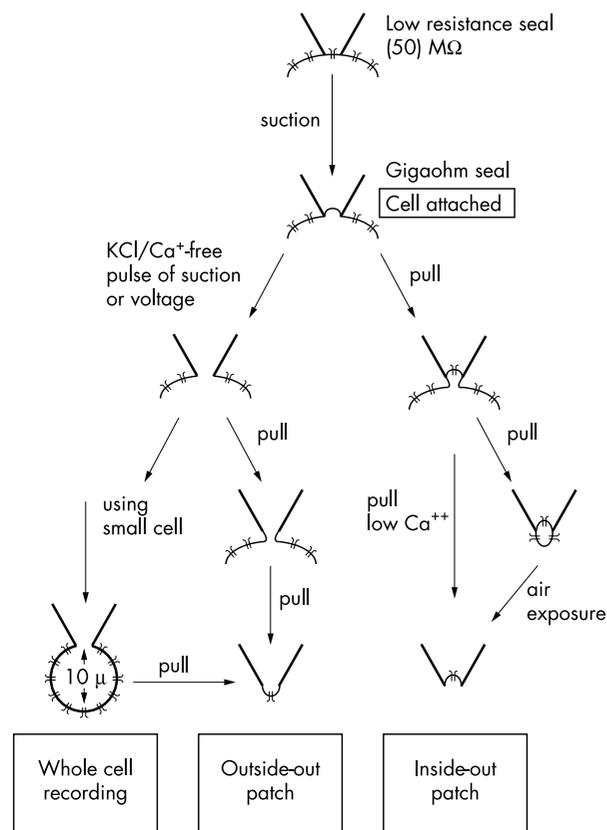
Figure 3 Schematic representation of the procedures for various recording configurations. A fine tipped (about 0.5 to 5 μm in tip diameter) glass patch electrode is used as a current monitor and the voltage in the pipette is held at a desired level. The first step in applying the technique is the formation of a high resistance seal between the patch electrode and the surface of the cell. Once the seal is established, several recording configurations are available to the investigator, and these fall into two broad categories. On the one hand, current flow through the patch of membrane under the electrode tip can be monitored, in which case single channel currents are usually recorded. Alternatively, for whole cell recording, the patch of membrane can be disrupted so that the electrode monitors current flow through the entire cell surface. The symbol ⫴ is inserted for easier recognition of the orientation of the membrane and the channels. Modified after Sakmann and Neher, 1985.[6]

Figure 4 Whole cell (A, C) and single cell (B, D) recordings of sodium currents from normal (A, B) and mutant (C, D) sodium channels. (A), (C): Representative whole cell current from transfected HEK 293 cells held at a potential of −85 mV. After a 15 ms prepulse to −120 mV the currents were elicited by an above shown test pulse to −20 mV. (B), (D): The downward deflections of the six subsequent current traces correspond to single channel openings (note the name *myotonia*, namely potassium aggravated myotonia).[7][8]
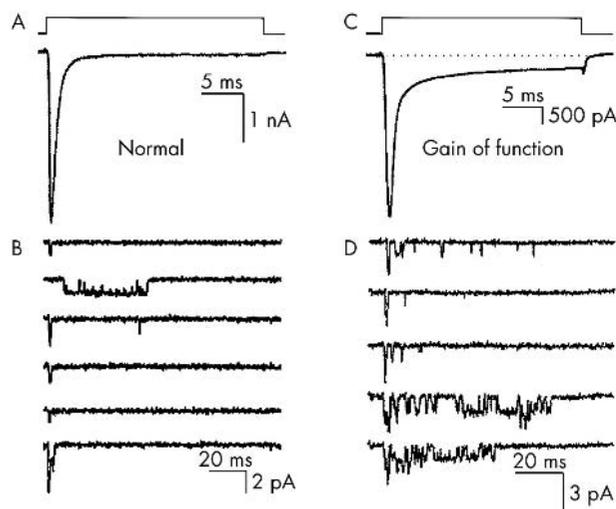
clamp technique is used to characterise the function of a channel ensemble or a single protein molecule.

By combining this functional expression with fluorescence microscopy or other microscope techniques such as atomic force microscopy, structure–function correlations can be determined (see below).

APPLICATION TO CLINICAL NEUROLOGY
Molecular biology and patch clamping combined: the key to identifying pathogenesis

In the last 10 years, the combination of electrophysiological and molecular genetic investigations has led to the exploration of the growing family of diseases caused by mutations in genes encoding voltage and ligand gated ion channels, the so called channelopathies. Most channelopathies require a non-normal situation, a so called trigger, before they present with recognisable symptoms, and only a few lead to permanent disability—for example, episodic ataxia type 2 or hypokalaemic periodic paralysis. Typically the symptoms occur as episodic attacks lasting from minutes to days, which show spontaneous and complete remission, onset in the first or second decade of life, and—for an unknown reason—

amelioration after the age of 40 or 50. Channelopathies are found in very different tissues such as brain, muscle, and secretory tissue.[9] Skeletal muscle was the first tissue in which such diseases—namely the myotonias and periodic paralyses—were recognised as ion channelopathies. Examples of neuronal channelopathies are episodic ataxia, familial hemiplegic migraine, and various types of epilepsy (see table 1). Channelopathies are also important models for more frequent disorders of non-monogenic aetiology.

For an understanding of the pathology of a given hereditary disorder, knowledge of the mutated gene is only a first step. More revealing is the detection of the functional defect that is brought about by the disease causing mutation. Much progress on the road to this aim was achieved by the combination of molecular biology and electrophysiological patch clamp techniques.

The channel pathology of two diseases is given as an example, one caused by gain-of-function mutations in a sodium channel, the other by loss-of-function mutations.

Myotonia

An example of a gain-of-function mutation is myotonia. The term ''myotonia'' designates involuntary muscle contractions that are caused by membrane hyperexcitability of skeletal muscle and which lead clinically to slowed muscle relaxation following voluntary muscle contractions. Myotonia is felt as uncontrolled temporary muscle stiffness by the patient.

The voltage gated sodium channel is essential for the generation of an action potential. Its upstroke is mediated by opening the channels that passively conduct a fast sodium inward current in a feed forward mechanism along both an electrical and a concentration gradient. Owing to the resulting high conductance of the membrane for sodium ions, the membrane suddenly depolarises from the resting value of −84 mV to approximately +25 mV. Immediate repolarisation of the membrane to the highly negative resting value is made possible by fast inactivation of the sodium channels, resulting in the usual predominance of the membrane conductance for potassium ions that may be further supported by opening of additional voltage gated

Table 1 Hereditary channelopathies of excitable tissues

Gene	Locus	Channel protein	Disease	Inheritance	Change
Cardiac muscle					
KCNQ1	11p15.5	Potassium channel α subunit, KCNQ1, KVLQT1	Long QT syndrome 1 Jervell and Lange-Nielsen	Dominant Recessive	Loss Loss
HERG	7q35-36	Potassium channel α subunit, HERG, eag related, Ikr	Long QT syndrome 2	Dominant	Loss
SCN5A	3p21	Sodium channel α subunit	Long QT syndrome 3 Brugada syndrome	Dominant Dominant	Gain Loss
KCNE1	21q22	β subunit of KVLQT1, MinK	Long QT syndrome 5	Dominant	Loss
KCNE2		β subunit of HERG, MiRP1	Long QT syndrome inducible	Dominant	Loss
RYR2	1q42-43	Ryanodine receptor 1, calcium release channel type 2	Catecholaminergic ventricular fibrillation	Dominant	Gain
CASQ2	1p13-11	Calsequestrin type 2, fast twitch	Catecholaminergic ventricular fibrillation	Recessive	Gain
Skeletal muscle					
SCN4A	17q23.1-25.3	Voltage gated sodium channel Nav1.4 α subunit	Hyperkalaemic periodic paralysis Paramyotonia congenita Potassium aggravated myotonia Hypokalaemic periodic paralysis 2	Dominant Dominant Dominant Dominant	Gain Gain Gain Loss
CACNA1S	1q31-32	Voltage gated L-type calcium channel α1 subunit, dihydropyridine (DHP) receptor	Hypokalaemic periodic paralysis type 1 Malignant hyperthermia type 5	Dominant Dominant	Unclear Unclear
RYR1	19q13.1	Ryanodine receptor 1, calcium release channel	Malignant hyperthermia type 1 Central core disease	Dominant Dominant	Gain Gain
KCNJ2	17q23-24	Voltage insensitive potassium channel α subunit Kir2.1	Andersen's syndrome, long QT syndrome 7	Dominant	Loss
CLCN1	7q32-qter	Voltage gated chloride channel ClC1 Altered splicing	Thomsen myotonia Becker myotonia Myotonic dystrophy type 1 Myotonic dystrophy type 2	Dominant Recessive Dominant Dominant	Loss Loss Loss Loss
ATP2A1	16p12	SERCA 1a, Ca-ATPase of sarcoplasmic reticulum, fast twitch 1	Brody's syndrome	Dominant, recessive	Loss
Endplate					
CHRNA1	2q24-32	nAChR α1 subunit	Congenital myasthenic syndrome	Dominant and	Gain and loss
CHRNB1	17p12-11	nAChR β1 subunit	(nAChR = nicotinic acetylcholine	recessive	of function
CHRND	2q33-34	nAChR δ subunit	receptor)		
CHRNE	17	nAChR 1 subunit			
RAPSN	11p11	Rapsyn, AChR associated protein	Congenital myasthenic syndrome	Recessive	Loss
Central nervous system					
SCN1A	2q24	Nav1.1 sodium channel α subunit 1	Generalised epilepsy with febrile seizures plus		
SCN1B	19q13.1	β1 subunit	(GEFS+2)	Dominant	?
SCN2A	2q23	Nav1.2 sodium channel α subunit 2			
SCN2A	2q23	Nav1.2 sodium channel α subunit 2	Benign familial neonatal/infantile convulsions	Dominant	?
KCNA1	12p13	Potassium ch α1 subunit, A-type, Kv1.1	Episodic ataxia type 1, partial epilepsy (?)	Dominant	Loss
KCNQ2	20q13.3	Voltage gated potassium channel α subunit Kv7.2	Benign familial neonatal convulsions Neuromyotonia	Dominant Dominant	Loss Loss
KCNQ3	8q??	Voltage gated potassium channel α subunit Kv7.3	Benign familial neonatal convulsions	Dominant	Loss
CACNA1A	19p13.1	Calcium channel P/Q-type α1 subunit	Episodic ataxia type 2 Familial hemiplegic migraine 1 Spinocerebellar ataxia type 6 Absence epilepsy	Dominant Dominant Dominant Dominant	Loss Loss Loss Loss
CACNA1F	Xp11.23	Calcium channel retinal L-type α1 subunit	Congenital stationary night blindness (CSNB2)	Recessive	Loss
CLCN2	3q26	Voltage gated chloride channel ClC-2	Idiopathic generalised epilepsies	Dominant	Loss or gain
CACNB4	2q22-23	Calcium channel L-type β4 subunit	Generalised epilepsy Episodic ataxia 3	Dominant Dominant	Gain Gain
CHRNA4	20q13.3	Nicotinic acetylcholine receptor α4 subunit	Nocturnal frontal lobe, ADNFLE	Dominant	Loss
GLRA1	5q31.2	Glycine receptor α1 subunit	Hyperekplexia = startle disease = stiff baby syndrome (STHE)	Dominant Recessive	Loss Loss

potassium channels (see second example). Gain-of-function sodium channel mutations—for example, those situated in the inactivation gate that is shown in fig 2 at the intracellular side of the channel protein—lead to an incomplete and destabilised channel inactivation that results in frequent channel reopenings and channel bursts, and hence in a pathologically increased sodium current (fig 4C, 4D). This persistent sodium ion influx generates repetitive muscle action potentials and thus myotonia.

If the sodium ion influx through mutant channels is large, an associated sustained membrane depolarisation may lead to a secondary loss of function of the 50% of sodium channels that are genetically normal. This loss of function can result in episodic weakness potentially occurring in myotonic conditions such as hyperkalaemic periodic paralysis and paramyotonia congenita. The patients are heterozygous for mutant and wild-type channels, but by dictating a change in cell excitability, the mutation results in a dominantly inherited disease.

Benign familial neonatal convulsions

Benign familial neonatal convulsions (BFNC) is the second example. BFNC is a rare dominantly inherited epileptic syndrome characterised by frequent brief seizures within the first days of life that typically disappear spontaneously after weeks to months. The disease is caused by various mutations in KCNQ2 or KCNQ3—voltage gated potassium channels that form a heterotetrameric channel complex which is slowly activated by membrane depolarisation.[10][11] Once opened, both channels conduct potassium ions from inside to outside along the concentration gradient and against the electric field. This outward current is the so-called "M-current" known to play an important role in the regulation of the firing rate of neurones.[12] It stabilises the highly negative membrane potential and reduces the neuronal excitability.

BFNC-causing mutations in KCNQ channels are clustered in two regions of the protein: in the P loop between segments S5 and S6 constituting the pore region, and in the C-terminus that is unusually long in all KCNQ channels (see fig 1). All mutations drastically reduce or abolish the M current, the pore mutations probably by affecting the ionic conductance,

Figure 5 Functional consequences of a mutation in KCNQ2, the Kv7.2 voltage gated potassium channel α subunit domain formed by 6T/1P. Several mutations in this domain cause benign neonatal familial convulsions (BNFC). The mutation, 2513delG, is a single base pair deletion that is located just seven codons before the regular stop codon and induces a frame shift, thereby resulting in a change in the last seven amino acids of the normal protein and in a prolongation of the mutant protein by another 56 amino acids before the new stop codon. The mutant KCNQ2 cDNA was functionally expressed in *Xenopus* oocytes. The mutation reduces the potassium current to less than 10%, as seen in the raw current traces in (A) (note the 10-fold higher current resolution of the mutant channel (noise reduced by filter) compared with the wild type channel, WT) and for the average of many such experiments in (B). Coexpression of both normal (WT) and mutant channels did not result in significantly less than 50% of WT current, suggesting there was no dominant negative effect of the mutation.[11]

and the C-terminus mutations by reduced assembly to heteromeric channels. Although it is now clear that the mutations exert loss-of-function at the channel level (see fig 5A, 5B) and thereby increase the excitability of the neurones that express the mutant genes, the question remains as to why the mutations result in seizures preferentially during the neonatal period. One possibility is that the premature brain is generally more likely to develop seizures than later in life.[13] Another explanation might be a differential expression of potassium channels during maturation in the hippocampus, leading to a dominant role of KCNQ channels in central neurones within the first days to weeks of life.[14]

As a result of the discovery of the pathogenesis of BNFC, a novel approach to the treatment of epilepsies emerged from identifying retigabine as an activator of M currents. Retigabine shifts the voltage dependence of steady state activation of these channels by about 20 mV in the negative direction, so that they are open at the resting membrane potential. This high channel-open probability "clamps" the neurones to resting potentials near to the potassium reversal potential.[15]
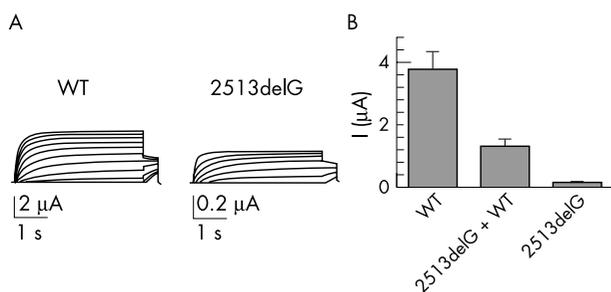
Knowledge of the functional defect that is brought about by the disease causing mutation is essential for an understanding of the aetiology and diagnosis of a given hereditary disorder. A unique feature is the availability of technical possibilities to study precisely the pathology of channel proteins by combining modern molecular biology with the patch clamp technique.
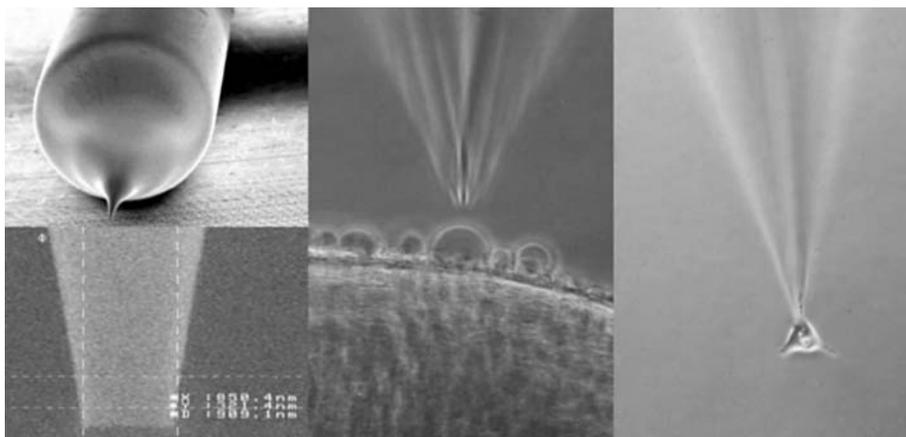
PRINCIPLES BEHIND THE TECHNIQUES
Channel specific techniques

Classical voltage clamp is done with two intracellular "sharp" microelectrodes (tip diameter <0.1 μm, resistance ~10 megohm when filled with 3 M KCl), one for clamping the membrane potential to values according to various pulse protocols for activation, inactivation, and so on; the other measuring the current conducted during the voltage steps. The advantage of this technique, which is still used, particularly for oocytes, is its simplicity. The disadvantage is that the membrane of larger cells and oocytes may not be homogeneously clamped to a certain value, which is especially problematic if rapidly activating ionic currents are studied.

Whole cell recordings are done with a glass pipette pulled as a "patch clamp microelectrode" (fig 6 left; tip diameter of ~1 μm, resistance of ~1 megohm when filled with 100 mM KCl). Once the seal is established, a brief suction is applied to the interior of the electrode in order to rupture the patch of membrane under the electrode tip. After this break in, there will be a low resistance pathway for current and diffusional flow between the electrode and the cell interior. The cell membrane is voltage clamped at the pipette potential by virtue of this low resistance pathway, and the electrode monitors the current flowing across the entire cell surface. The ionic composition of the cell cytoplasm rapidly equilibrates with the pipette contents, offering a pathway for the control of cellular constituents. Although whole cell recording can be highly accurate, two important limitations are associated with the technique. The first is connected with the resistance in series with the membrane (R_s), which in whole cell recording is the access resistance between the interior of the pipette and the cell cytoplasm.[16] Another limitation of the whole cell configuration is that important intracellular regulatory molecules—such as cAMP, Ca^{2+}, or GTP—can diffuse out of the cell through the patch electrode; thus the physiological regulation of these important second messenger substances is disrupted during whole cell recording. The *perforated patch* technique[17] provides a solution to this problem

Figure 6 Typical microelectrode suited for patch clamping (left panel, courtesy of Dr W Ebert) and its attachment to either a membrane bleb formed from a native human skeletal muscle fibre (middle panel) or an HEK cell (right panel). The upper left panel shows the glass microelectrode, the lower part the tip, with a diameter of about 700 nm. The plasmalemmal bleb in the middle panel was formed by careful stretching of native muscle fibres at $\geqslant 10^{-5}$ M [Ca^{2+}] in the bathing solution. The membrane of native HEK and other cultured cells is so clean that giga-ohm seals can easily be produced without additional measures.

by making it possible to record macroscopic currents with a cell-intact recording configuration. This configuration is obtained by including a pore forming antibiotic, such as nystatin or amphotericin B, in the pipette solution. After a seal is formed on the cell, the antibiotic channels insert in the patch of membrane under the electrode tip, thereby providing electrical continuity between the pipette and the cell interior.

In conclusion, the whole cell mode measures the current through the total cell membrane superimposed with noise. This ''macroscopic'' current corresponds to the average of many simultaneously conducting channels and therefore resembles the intracellular recordings. Because of the simple and fast analysis, it is the configuration that is most frequently used.

Cell attached, *inside-out*, and *outside-out* modes allow the measurement of a small number of channel proteins or even a single channel in the electrically isolated membrane patch, depending on how many channels are embedded in the patch. The various configurations are available to the investigator once the seal is established (see fig 3). The *cell attached* mode is already made as soon as a giga-ohm seal between the patch electrode and the cell membrane is established (fig 6, left: same electrode as for whole cell recordings, but filled with an ''extracellular'' solution). The background noise can be sufficiently attenuated so that the current flowing through a single ionic channel can be resolved (single channel recordings).[4] A special form is the bleb attached mode that enables the measurement of adult native cells without enzymatic treatment (fig 6, middle). Blebs (or blisters) can be formed—for example, by stretching skeletal muscle fibres in a high calcium bath solution.[18 19] The advantage is that the plasma membrane is absolutely clean so that a giga-ohm seal can be achieved relatively easily.

If the recording electrode is withdrawn from the cell after a cell-attached patch is formed, the patch of membrane can be excised from the cell with the inside surface of the membrane facing the bath solution; this is called the *inside-out configuration*. An *outside-out* patch can be formed by removing the electrode after entering the whole cell configuration. One advantage of these cell-free patch recordings is that they provide more accurate control of the membrane potential. In the cell attached mode, the membrane potential of the patch is equal to the resting potential of the cell minus the pipette potential. As the resting potential may not be known with

certainty or might vary during an experiment, there will be uncertainty in the value of the patch membrane potential. However, in the isolated patch configurations, the magnitude of the patch potential is equal to the pipette potential and is therefore known with precision. A second advantage is that
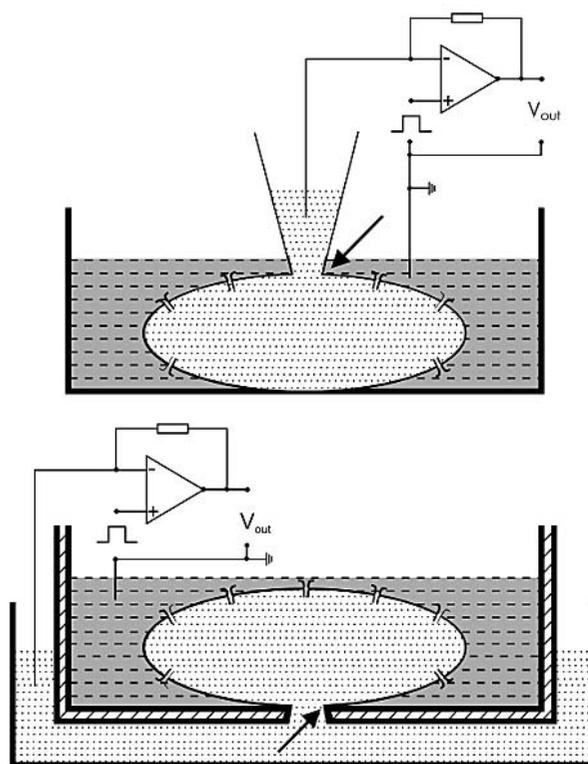
Figure 7 Patch clamp arrangements. Upper panel: classical whole cell configuration and circuit using a glass microelectrode. Lower panel: patch clamp chip and circuit. This arrangement can be multiplied and automated for high throughput screening. In addition, the arrangement allows the scientist to install a second technique—for example, fluorescence or force microscopy—from the upside of the setup.

the solution bathing one surface of the patch can rapidly be changed simply by changing the bath solution.

Once a recording mode is established the recordings can be made with a typical patch clamp circuit which uses the electrode both to control the potential and to measure the currents conducted by the channels (fig 7, top).

Additional cloning techniques
Transient expression in oocytes and transient and permanent expression in heterologous cells

Oocytes from *Xenopus laevis* have become a widely used preparation for the expression of cloned ion channel genes. A series of recent papers has reviewed many of the technical aspects of the use of this expression system.[20–25] Isolated *Xenopus* oocytes are capable of translating injected mRNA from a variety of sources and of leading to a high channel density in the membrane and large ion currents. The mRNA can be extracted from the tissue of interest[23] or it can be synthesised from an isolated clone (cDNA) which codes for the channel protein.[24] The isolated mRNA is pressure injected into the oocyte or its nucleus through a relatively large diameter (10 µm) microelectrode. Within a few days, functional channels will be present in the surface membrane of the oocyte and can be studied with various voltage clamp techniques, including a conventional two microelectrode voltage clamp or single channel patch clamp recording.[25] Several types of ionic channel have now been expressed in oocytes using these techniques.[26 27]

In contrast to transient expression systems, stable expression involves the introduction of exogenous DNA into the genome of a cell so that it is transcribed with the cell's DNA and inherited by offspring cells during cell division. One of the obvious advantages of this type of system is that once the cell line is established, studies can be performed on a uniform cell population without re-establishing expression for each experiment (for an example see a patched HEK cell in fig 6, right panel). The foreign DNA can be introduced by microinjection, electroporation, viral expression vectors, or transfection using various techniques.[28]

### POTENTIAL APPLICATION OF THE TECHNOLOGIES IN THE FUTURE
High throughput screening for neuroscience and pharmaceutical industry

Although very powerful, the patch clamp technique is extremely labour intensive and thereby limited to a throughput of 10–20 individual cell measurements a day. Thus several firms are trying to develop technologies for high throughput screening. Achieving this goal requires more than just the automation of existing patch clamp techniques; it requires the development of an entirely new paradigm for making electrophysiological measurements. All developments are based on positioning a cell on a small pore separating two isolated fluid chambers in a manner that requires no manual intervention or micromanipulation (fig 7, bottom). In order to perform whole cell electrophysiological measurements within this geometry, two criteria must be met. First, a high resistance seal must form between the cell membrane and peripheral region of the substrate pore. As in the case of patch clamp electrophysiology, this ensures that the current measured between the two electrodes passes through the cell membrane. Second, in order to be able to control the cell's membrane potential, a low resistance electrical pathway must form through the cell wall that covers the pore. This latter requirement in effect places the associated electrode at the interior of the cell and allows one to clamp the membrane potential over the rest of the cell membrane. Once these criteria have been met, and assuming no manual intervention, it is then possible to

conceive a parallel format where many wells can be measured simultaneously.

Functional testing of tissue specific channel splice variants

Even though the human genome has been sequenced, it is not completely clear which genes are expressed in neurones and how these are tissue specifically spliced. In particular, the functional alterations of the splice products are almost unknown up to now. It may still take several years before more precise knowledge of the exact interplay between these proteins and other regulatory or signalling pathways is available.
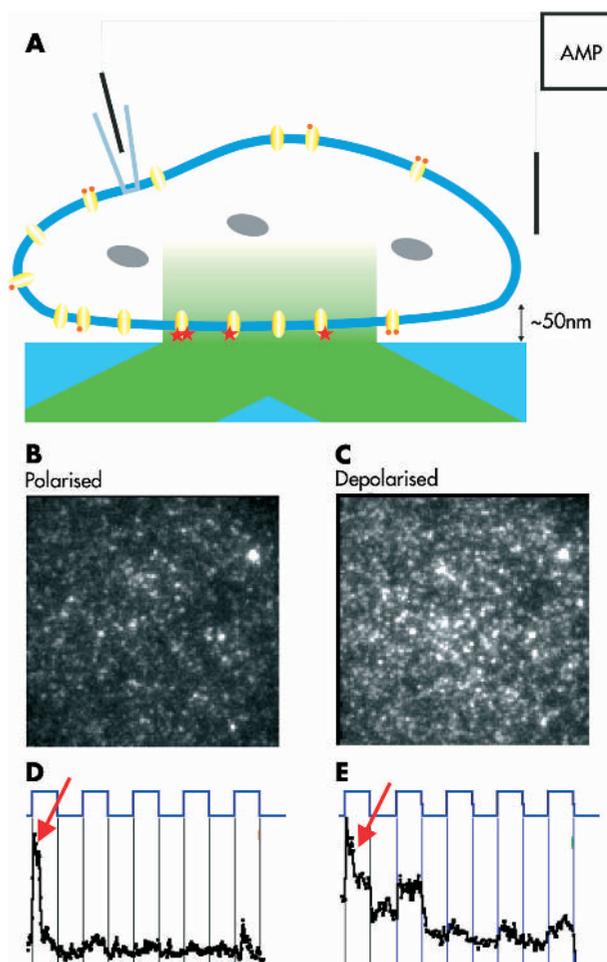
Figure 8 Imaging of single gating charge movements. (A) Principle of TIRFM on channel proteins. (B), (C) CCD images off fluorescence signal from a 20×20 µm membrane patch from a *Xenopus* oocyte transfected with a shaker potassium channel labelled with a tetra-methyl-rhodamine-maleimide (TMRM) at the voltage sensing S4 domain. Individual spots correspond to single TMRM molecules attached to S4. Upon membrane depolarisation, an increase in fluorescence can be observed. (D), (E) Typical time traces of individual spots showing the response of a single voltage sensing S4 domain to depolarisation and subsequent photobleaching (red arrow) during the first depolarisation (an indication for a single flurophore attached to a single S4) (D). (E) Example of another spot, where bleaching of one TMR occurs during the first depolarisation, while the second flurophore bleaches only after the second depolarisation. Measurements of single gating charges are not possible by electrical means because of the low signal. Therefore the attachment of TMRM to the voltage sensing S4 domain presents the only practical way to detect single gating charge movements.[29] AMP, amplifier.
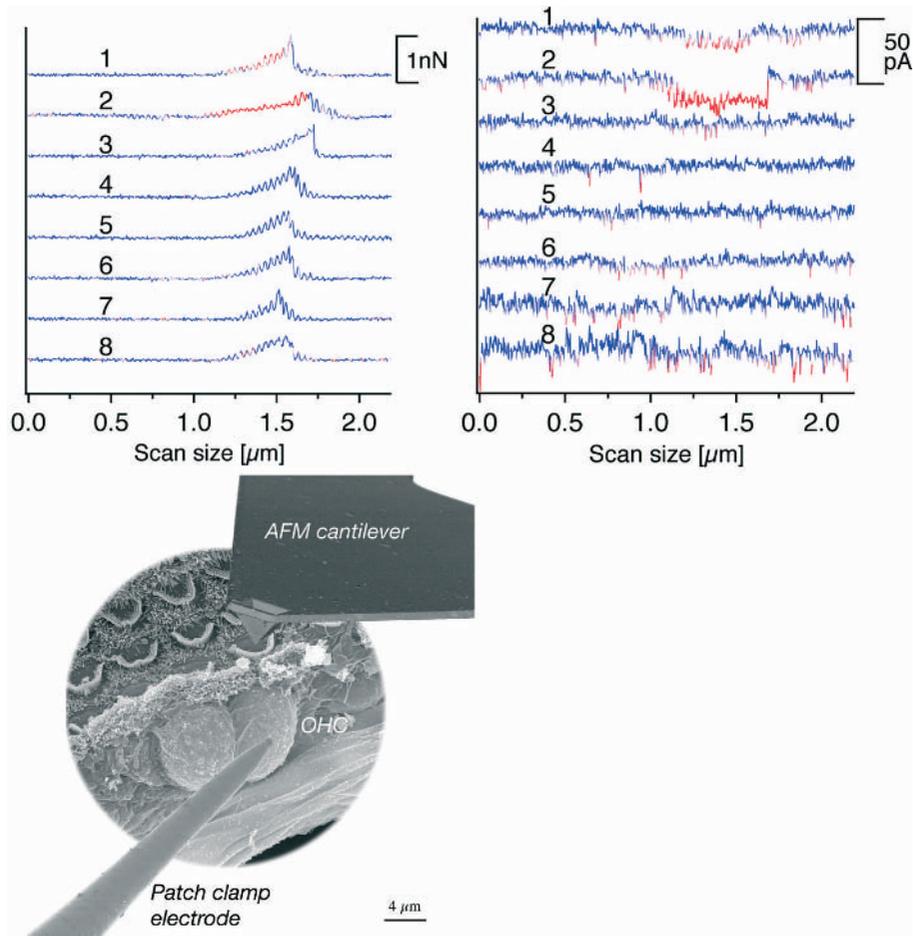
Figure 9 Force and current responses to displacement of single stereocilia of postnatal rats. Upper left panel: eight adjacent stereocilia of a cochlear hair bundle are individually displaced, scanning an atomic force microscopy (AFM) tip in excitatory direction. Interaction between each stereocilium of the tallest row of stereocilia with the AFM tip results in an increasing force bending the AFM cantilever upward. After losing contact, the AFM force sensor moves down to its initial position. An additional sinusoidal modulation (f = 357 Hz) is added to the lateral movement of the AFM force sensor. Upper right panel: an in-phase current response (opening and closing) of the mechanosensitive transduction channel was observed for stereocilia Nos 1 and 2 until the transduction current through the channel pore reaches saturation where the channel is in the maximum open state (see traces 1 and 2). Although the maximum forces for the remaining six stereocilia are of the same magnitude, no clear response was observed. The last trace (No 8) shows an increased noise level, which is caused by an increased leak between patch clamp pipette and cell membrane. Lower panel: experimental setup showing the anatomy of the AFM cantilever, a partition of an organotypic culture of the organ of Corti with the three rows of outer hair cells (OHC), and the patch clamp electrode.[44 46]

Pharmaceuticals and patch clamping combined
In vitro testing of drugs for channels
The electrophysiological study of mutant channels expressed in cell systems allows one to characterise the functional alterations and to develop new strategies for the treatment of ion channelopathies—for example, by testing drugs that are already on the market for other indications; or by testing drugs that could be designed specifically either to block mutant channels that reveal a gain-of-function, or to activate non-mutant channels that could compensate for channels functionally lost by a mutation.

In vitro testing of drugs for neuroprotection
Part of the energy demand of neurones is required for active ionic pumps that compensate for the passive flux of ions through specific and non-specific ion channels along the diffusion gradients. A reduction of the transmembrane ionic flow diminishes the energy demand of neurones considerably. The pharmacological modification of the state of voltage or ligand activated ion channels thus provides potentially powerful strategies for neuroprotection.

TIRF microscopy and patch clamping combined: improving structure–function relations
Total internal reflection fluorescence (TIRF) microscopy is a powerful technique for visualising fluorescently labelled membrane proteins. The evanescent wave can selectively excite fluorescent molecules in or near to the cell membrane. TIRF microscopy is particularly suited for the combined study of voltage sensor movements and the resulting transmembranous gating and ion currents (fig 8A). Structural changes of channel ensembles can be studied in real time using a photomultiplier. Conformational changes of several single channels can be detected simultaneously by spot-like signals in the cell membrane changing their fluorescence intensity. The temporal and spatial resolutions of the changes are restricted by the characteristics of the detecting ccd camera.

In studies undertaken by Sonnleitner et al,[29] a structural change of the gating subunit of an ion channel results in a change in the fluorescence intensity of the attached fluorophore (fig 8B 8C). Hence the attached fluorophore acts as a sensor for structural changes in the ion channel that can be directly related to ion channel function through electrical

measurements (fig 8D, 8E). TIRF microscopy can also be used in conjunction with FRET or other methods to detect relative movements between channel parts. This innovative combination of techniques allows molecular resolution of small motions underlying ion channel activation—for example, by ligands—and will probably find widespread use in the study of membrane associated molecules.[30] TIRF can also be combined with FRAP, a method that allows one to study the mobility of transmembrane proteins.

Atomic force microscopy and patch clamping: characterisation of mechanosensitive channels

Formerly, atomic force microscopy (AFM[31]) and other scanning probe microscopies were applied exclusively to hard materials where the surface needed to be analysed—for example, for topographical, electrical, magnetic, thermal, and elastic properties and friction. About 10 years ago, these modern nanotechnologies also began to be used for the characterisation of soft and moist surfaces, such as the surface of living cells and biomembranes.[32–34] AFM is increasingly used to study the elastic and plastic properties of the cytoskeleton of cultured cells,[35–38] to image neurones and glia directly,[39] and even to study the binding forces between individual molecules and their respective subunits.[40 41] Studies of docking and fusion of synaptic secretory vesicles at the neurone membrane allow us to determine the proteins that make a principal contribution to the interactive binding force between the two membranes.[42] By using atomic force microscopy, laser confocal microscopy, electrical recording, and biochemical assays, the molecular conformations of reconstituted globular amyloid β protein (AbetaP) as well as their real time and acute effects on neuritic degeneration have been studied. AFM of AbetaP1-42 reconstituted in a planar lipid bilayer revealed multimeric channel-like structures, and electrophysiological recordings demonstrated the presence of multiple single channel currents of different sizes.[43]

AFM has also been combined directly with the patch clamp technique for the characterisation of biological mechanoelectrical transduction channels in living inner and outer hair cells of the cochlea.[44–46] Using an AFM stylus with a tip diameter of only a few nanometres, it was possible to displace individual stereocilia of cochlear hair cells, resulting in opening of single transduction channels. In contrast to the outside-out and the inside-out patch clamp configuration, this technique allows investigation of single mechanosensitive ion channels in entire cells (fig 9).

CONCLUSIONS
Methods that help to determine the electrical properties and the structure–function relations of neuronal channels are becoming increasingly adapted to study single proteins in their native environment. This is the basis for understanding the pathological mechanisms of the channelopathies and for developing new treatment strategies in the future.

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APPENDIX 1
Glossary of terms
Terms related to channel
- *Channelomics* is the short term for proteomics of voltage and ligand gated ion channel proteins of a cell and includes all aspects of an ion channel protein such as transcription, expression, and imprinting of the channel encoding genes; splicing and editing of the RNA; translational and post-translational effects on proteins and subunit composition; selectivity and gating of the channel pores; and modulatory effects of mutations, drugs, and toxins on the structure and function of the channels.
- *Channelopathies* are defined as disorders caused by pathology of ion channel *function*. These may be due to mutations in the corresponding genes or to binding of antibodies and toxins to the channel protein.

Terms related to patch clamping
- *Cell attached patch*: recording microelectrode sealed onto the cell, current flowing through the channels (usually one to 10) situated in the membrane patch can be resolved.
- *Whole cell patch*: after a cell attached patch is formed, a brief suction is applied to the interior of the electrode in order to rupture the patch of membrane under the electrode tip; after this break in, there is a low resistance pathway for current and diffusional flow between the electrode and the cell interior; the cell membrane is voltage clamped at the pipette potential by virtue of this low resistance pathway, and the electrode monitors the current flowing across the entire cell surface; the ionic composition of the cell cytoplasm rapidly equilibrates with the pipette contents, offering a pathway for the control of cellular constituents.
- *Inside-out patch*: electrode is withdrawn from the cell after a cell attached patch is formed; the patch can be excised from the cell with the inside surface of the membrane facing the bath solution.
- *Outside-out patch*: electrode removed after entering whole cell configuration.

Terms related to force microscopy
- *AFM* (atomic force microscopy) uses a mechanical probe to magnify surface features up to 100 000 000 times, and produces three dimensional images of the surface of rigid and soft materials. A fine microfabricated tip (<30 nm diameter) of a cantilever is brought into contact with the sample and scanned. The forces between the tip and the sample are used to image the surface. The interaction forces can be as low as the force needed to unwind a single coiled molecule. The dynamic modes allow visualisation of processes with a time resolution in the millisecond range.

Terms related to fluorescence microscopy
- *Emission*: All fluorescent dyes emit light of one wave length (for example, green) after they have absorbed light of another wave length (for example, blue). However, if a very high intensity blue light is delivered to the dye, the dye will ''photobleach,'' meaning that the high intensity light has rendered the dye unable to fluoresce.
- *FRET* (fluorescent resonance energy transfer): non-radiative transfer of photon energy from an excited fluorophore (the donor) to another fluorophore (the acceptor) when both are located within close proximity (1–10 nm); the technique can resolve the relative proximity of molecules beyond the optical limit of a light microscope

- *FRAP* (fluorescence recovery after photobleaching): after high intensity light had rendered the dye unable to fluoresce, surrounding molecules that have not been photobleached migrate into this blackened area and can be made visible. Used to determine whether a protein is able to move within a membrane or is tethered to structural components of the cell

- *TIRF* (total internal reflection fluorescence) microscopy allows visualisation of fluorescently labelled membrane proteins. The excitation light beam penetrates only a short distance when totally internally reflected at the interface plane. Depending on the angle of the excitation beam and the refractive index ratio, the penetration depth may vary between 50 and 300 nm. This surface electromagnetic field, called the "evanescent wave," can selectively excite fluorescent molecules in or near to the cell membrane. Background fluorescence from fluorophores either in the extracellular solution or inside the cells is suppressed. Also, because TIRF minimises the exposure of the cell interior to light, the survival of the cell during imaging procedures is much enhanced relative to standard illumination, which penetrates the cell (epi- or transillumination).

APPENDIX 2
Associated websites of interest
http://www.essen-instruments.com
http://www.zeiss.de
http://www.uar.at
http://www.bio.davidson.edu
http://www.channelopathies.org/
http://beam.to/calcium_quark

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