

Automated Patch Clamp in Cystic Fibrosis Drug Discovery

A case study on how Enterprise Therapeutics successfully applied automated patch clamp in primary screening and lead optimization in its TMEM16A drug discovery programme



 **SOPHION** | QPatch 48X

In October 2020, **Enterprise Therapeutics’** TMEM16A programme was acquired by Roche/Genentech. This acquisition was the culmination of a research and discovery journey initiated by Enterprise Therapeutics in 2014, with the goal of providing a novel treatment paradigm suitable for patients with cystic fibrosis and other muco-obstructive diseases.

This journey also highlights the enormous technological progress that has developed in the performance and capabilities of Automated Patch Clamp (APC) platforms in the past two decades. The direct measurements of ion channel activity and accurate control of channel gating parameters that APC systems provide, proved pivotal in hit finding, hit triage and rapid lead compound optimization (Danahay et al. 2020).

In this whitepaper, we highlight how APC was instrumental in the success of the TMEM16A programme. Using this case study, we show how the development of APC systems in the past decade is opening up a new era of drug discovery, providing tools for both high throughput and high-quality ion channel recordings. Consequently, APC gives researchers the capability to advance their drug discovery on ion channels, allowing faster definition and screening of these critical disease targets, leading to wide, new investigative vistas in developing much-needed novel treatments.

Cystic Fibrosis: the need to find alternative treatments

Cystic fibrosis (CF) is a devastating disease, graphically described from the patient perspective as drowning from the inside. It is an inherited disorder that causes organ damage throughout the body, with particularly severe damage to the lungs and digestive system, impairing normal growth and development. In people with CF, a defective gene causes secretions in the lung and digestive tract to become sticky and thick. This leads to ducts, tubes and passages becoming blocked, causing tissue damage and increasing the risk of infections, especially in the lungs.

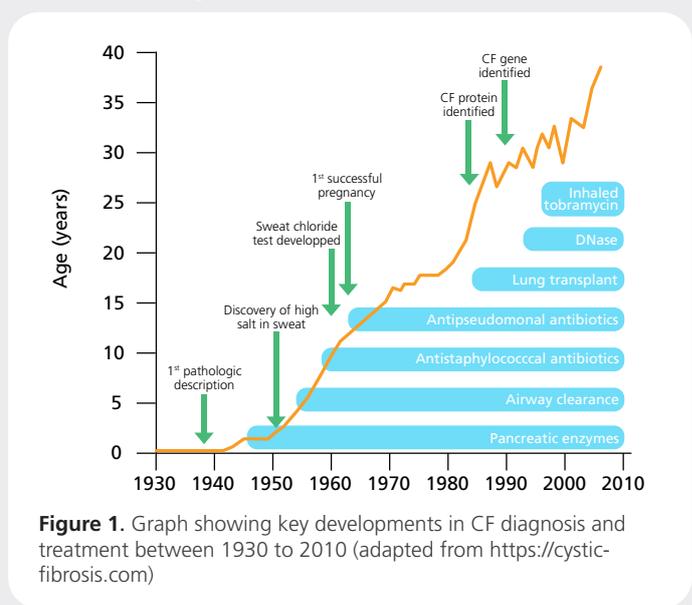
With an incidence of ~1 in 2500 births, CF is the most common lethal, genetic disorder of Caucasians. CF is a recessive disorder, with patients inheriting a copy of the defective Cystic Fibrosis Transmembrane Receptor (CFTR) gene from each parent. The incidence of defective CFTR genes is relatively high, with 1 in 20 people harbouring a CFTR re-

cessive gene mutation. Although these individuals, termed ‘carriers’, have reduced CFTR function, they are healthy and commonly display no symptoms of the disease.

Up until the 1940s-50s babies diagnosed with CF often died before the age of three. With the introduction of antibiotics in the 1950s-60s, life expectancy increased dramatically, yet patients would still only be expected to live into their second or third decade. New medicines introduced since then have mainly served to alleviate CF symptoms or slow the disease progression, with limited improvements to the patient’s quality of life. This was mainly achieved by antibiotic therapy combined with treatments to clear the thick mucus from the lungs. Collectively, these developments have increased life expectancy to ~40 years – see Figure 1.

However, even in the early 2000s, many patients living with CF still faced significant challenges, including frequent hospitalisations; complications such as CF-related diabetes and depression; and time-consuming treatment plans routinely taking 2-3 hours a day (www.cff.org).

In the last decade, Vertex has developed and introduced several successful CF therapies that restore function to the defective CFTR gene product (‘CFTR modulators’, Smyth 2020). These therapies have been transformational for many patients, delivering significant improvements in lung function and other clinical endpoints, and are expected to provide a considerable increase in life expectancy (Balfour-Lynn & King 2020). However, there are still many CF patients, ~10% of the population, for whom these therapies are not suitable, requiring novel and alternative treatment strategies.

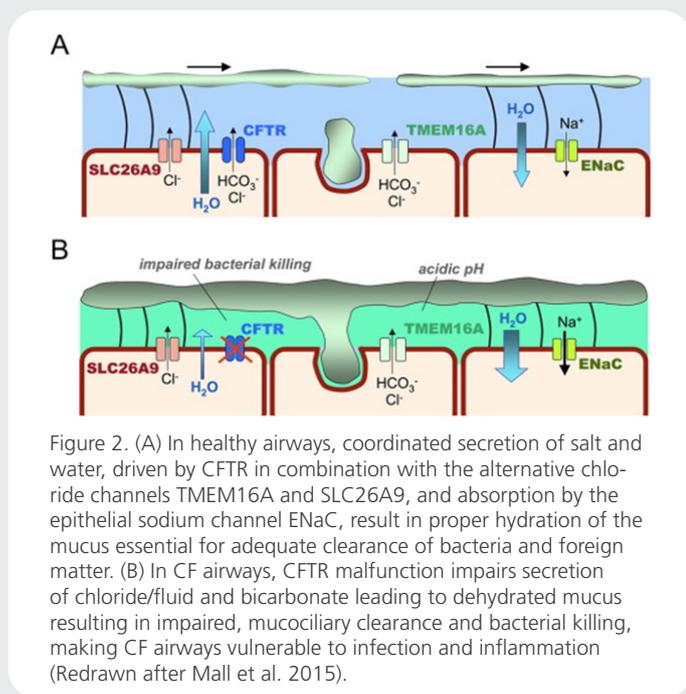


TMEM16A: an alternative CF target?

The CFTR gene encodes a cyclic AMP-regulated chloride channel, a pivotal protein responsible for epithelial chloride transport. In the lungs, chloride transport is vital in maintaining the hydration status of the airways such that mucus is runny and fluid, which fulfils a key role in protecting the airways from dangerous insults such as bacteria and other noxious particles, so it can be moved up and out of the lungs.

Mutations of the CFTR gene lead to defects in CFTR chloride ion channel function, resulting in dysregulation of epithelial fluid transport in the lung. This dysregulation leads to mucus becoming thick and sticky. The role of mucus is now compromised; it fails to clear bacteria and other foreign matter, leading to infection and inflammation, and can 'plug' the airways, further compromising lung function. More than 2000 cystic fibrosis-causing mutations have been described to date. Different variants and variant combinations (genotypes) are associated with varying disease symptoms (<https://cftr2.org/resources>).

CFTR is not the only chloride ion channel present in the airway epithelium – there is also a calcium-activated chloride conductance channel (CaCC). Although the presence of this channel has been known for decades, its molecular identity was only elucidated as TMEM16A (sometimes referred to as Anoctamin1 (Ano1)), in 2008 (Caputo et al. 2008, Yang et al. 2008, Schroeder et al. 2008). See Figure 2.



TMEM16A offers the opportunity to increase chloride transport in the airways, compensating for the defective flux through mutated CFTR (Li et al., 2017). This approach, sometimes referred to as a 'surrogate' or 'bypass' approach, is attractive for the following reasons:

1. TMEM16A has the potential to treat all CF patients regardless of their genotype ('mutation agnostic'), including the 10% not currently suitable for CFTR modulators.
2. Additional benefit may be delivered with in combination with CFTR modulators to further benefit patients.
3. TMEM16A offers an opportunity to treat non-CF diseases characterised by mucus congestion, such as chronic bronchitis.

Designing the Screening Study

To identify lead compounds which positively modulate TMEM16A, Enterprise Therapeutics adopted a parallel screening approach employing indirect cell-based fluorescent screens and direct electrophysiology screening using APC. The criteria for the APC electrophysiology screening assay were:

1. As TMEM16A is gated by the concentration of intracellular calcium ($[Ca^{2+}]_i$) and membrane voltage, both must be accurately controlled.
2. The ability to screen up to 20,000 compounds in a time and cost-effective fashion.
3. Stable and high-quality recordings, enabling detection of low-efficacy compounds.

Together, these criteria required a system capable of routinely achieving GΩ seals, and disqualified APC platforms that required seal enhancing solutions. Although seal-enhancing solutions effectively generate higher resistance seals, particularly with challenging cell types, these recording solutions combine high internal calcium with high external fluoride, making them unsuitable for TMEM16A. Also, the use of seal enhancers has other reported unwanted side-effects e.g. calcium fluoride gives rise to a non-linear and time dependent leak current (Lei et al. 2020).

At the time of TMEM16A APC screen initiation (2015/2016) there were no suitable screening assays on any of the 384 channel automated patch clamp platforms, due to the necessity of using calcium in the seal enhancing solutions; however, this has since been resolved using the 384-well APC platforms including the Qube 384 (Sauter, 2016).

Consequently, the logical choice of APC platform was QPatch, which is still the only marketed APC platform that can achieve $G\Omega$ seals in physiological intra- and extracellular solutions.

TMEM16A screen using QPatch 48

Running a 20k compound screen on a QPatch with 48 recording sites would have made the project both time-consuming and expensive. To compensate for the lower throughput (when compared to true high-throughput screening platforms), an ingenious assay design was developed as part of Enterprise's collaboration with the University of Sussex Drug Discovery Centre. Test compounds were added sequentially, six compounds per recording site, thus increasing throughput six-fold. This multiplexing assay was achievable since QPatch uses microfluidic channels to rapidly and efficiently exchange test solutions by complete wash-through of the recording site (Figure 3). This facilitates multiple, sequential additions of compounds. A positive control (a tool compound TMEM16A potentiator) was added at the end of each experiment. Extracellular solution (ECS) was used as the negative control solution to establish the initial baseline current.

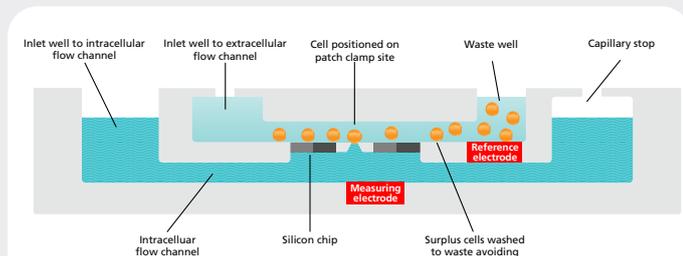


Figure 3. Cross section cut-away schematic of a QPatch recording well (one of 16 or 48 recording wells in a planar array). This shows the microfluidics inlets and throughflow waste reservoir for application of cell suspensions (orange spheres), intracellular (light blue) and extracellular solutions (slate blue) to the patch clamp recording site. In addition to the microfluidic flow channels, each recording chip also houses the reference (labelled red rectangle) and measuring electrodes (labelled red rectangle).

Briefly, the screening assay format was as follows (also see Figure 4):

- Extracellular solution (ECS) additions from the saline reservoir stabilise whole-cell configuration and determine the control current recording level.
- Six compounds, single concentration, were added sequentially (with a 5-minute incubation for each compound) from a 96 well compound plate/MTP.
- Following the 6 x 5-minute test compound additions, each recording had a potentiator reference compound (positive control) added from the reference reservoir.

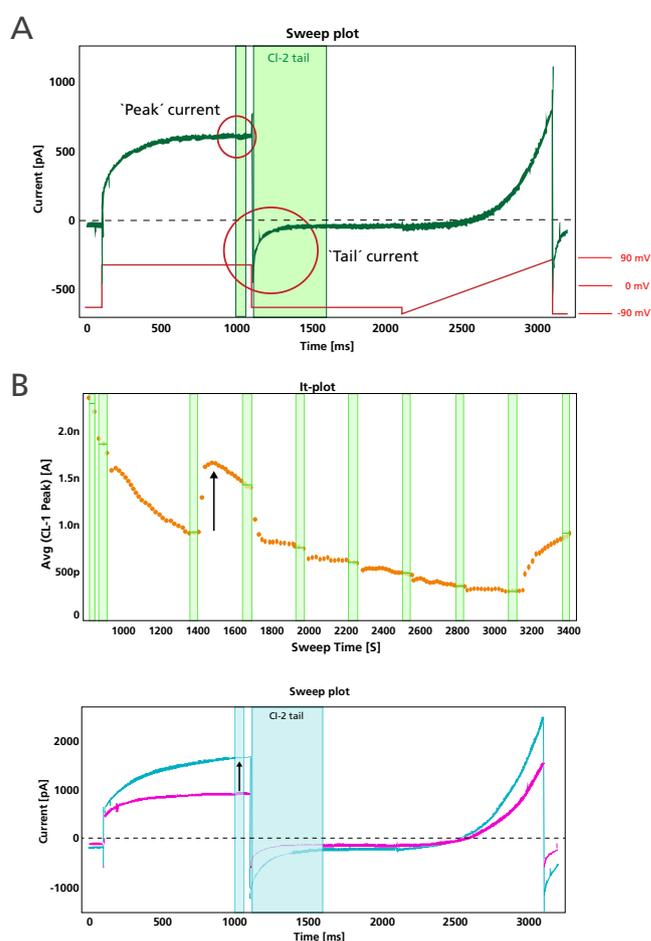


Figure 4. (A) An example whole-cell current trace (in dark green, left y-axis, pA) of TMEM16A activated by an EC_{20} of intracellular calcium combined with membrane depolarisation (the voltage pulse protocol is shown in the red trace, right y-axis, mV). Cursors (denoted by light green bands) measure the peak and tail currents elicited at each voltage pulse and were plotted as a current-time course plot (B) Example recording from a single screening well. **Top panel:** Current-time course plot (orange dots) for complete recording period. Each of the green bands indicates solution exchange: two ECS control applications, followed by 6 x 5 min test solution applications. In this example, the first application of test compound produced current potentiation and is a potential 'hit'. The subsequent five additions of test compound did not produce increases in current and were followed by a positive control reference at the end of the recording. **Bottom panel:** Sweep plot of potential hit compound. Current traces in ECS control solution (pink) and test compound potentiated current (turquoise).

The role of APC in lead optimization

In addition to a critical role in 'hit' finding, the QPatch TMEM16A assay also served a pivotal role in supporting lead optimisation for the TMEM16A potentiator programme. Two key factors drove this decision:

1. Throughput = speed: the assay's ability to screen 1000s of compounds per week, facilitated by the QPatch's unattended operation capabilities, ensured rapid delivery of data to the medicinal chemistry team. This was important to ensure fast iteration in compound design cycles, reducing lead optimization time.
2. Correlation = reduced resources: the benchmark assay for epithelial ion channel research is Ussing chamber ion transport using human cells expressing the native channel. However, this assay is highly labour intensive (cells requiring 3-4 weeks of culture), low-throughput, and uses precious resources (human cells) and expensive reagents. The excellent correlation of data from the QPatch assay to ion transport data reduced the need for routine ion transport assays, providing cost and time savings (Figure 5).

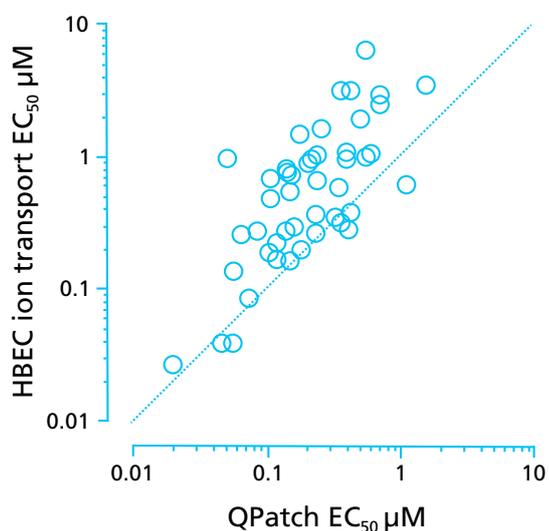


Figure 5. Strong correlation between QPatch assay and primary cell ion transport for TMEM16A modulator compounds. Data generated from recombinant channels expressed in an engineered cell line in the QPatch TMEM16A assay show a high correlation to Ussing chamber-generated data from primary human bronchial epithelial cells (HBEC).

Primary screening on a 48 channel APC platform – is that possible?

Screening of even 20k compounds can easily be done on a 48-channel platform when thinking outside the box and using the tools available. In this screen, test compounds were added sequentially, six compounds per recording site, thus increasing throughput six-fold. This multiplexing assay was achievable since QPatch uses microfluidic channels to exchange test solutions rapidly and efficiently.

Time and resource efficient use of automated electrophysiology to screen a 20k compound library for potentiators of TMEM16A:

- 20k compounds
- 10 workdays
- 1.5 FTE
- 2 QPatch 48
- 115 QPlates
- ~250 compound plates
- Minimum of $n = 1$ per compound

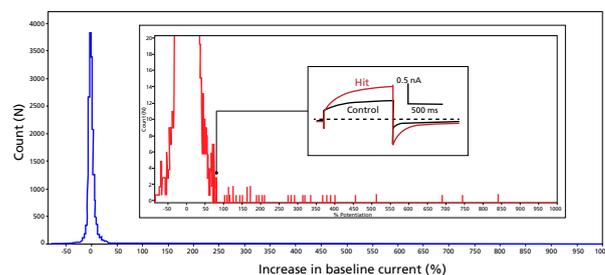


Figure 6: QPatch 20k screen performance summary showing distribution of activity of compounds screened - the centre of the distribution was -0.3% with a standard deviation of 19%. Using these criteria 0.98% of compounds screened were defined as 'hits'. Inset shows distribution with an expanded Y-axis, with the raw current data trace for one of the hit compounds highlighting the screen's ability to detect low efficacy compounds.

Advantages

- Fast and efficient.
- Accurate control of both intracellular Ca^{2+} and membrane voltage.
- Stability and reproducibility enabled good resolution of potentiator activity.
- High quality functional ion channel data at the single-cell level.
- No seal enhancers required for a calcium-sensitive target.

The changing role of APC in drug discovery

Since its introduction in the 2000s, APC has experienced a changing role in the drug discovery process. Widely introduced to meet the challenges of safety screening, particularly concerning the hERG channels, APC has moved to become a core resource to support ion channel drug discovery programmes, including screening campaigns.

In more recent years, a 'traditional' ion channel drug discovery screening cascade would employ a high-throughput non-electrophysiological primary screen, commonly fluorescence-based, to screen a large (>500k) compound collection. The output of this screen would then be triaged using APC to validate 'hits' and remove erroneous, false positives. Fluorescence-based assays for ion channel screening are high throughput and cost-effective to run but have well-described limitations outside of false positives. From a drug discovery perspective this is still a compromise as the characteristics that pre-

dispose fluorescence assays to false negatives are arguably the major concern. These include reliance on an indirect/non-linear readout of channel function, poor signal to noise and lack of control of the channel gating/state.

With improved performance and capabilities, the latest generation of APC solutions allow high throughput, whilst maintaining high quality (e.g. GΩ seals) and data rich ion channel recordings (Bell & Dallas, BJP, 2018), significantly reducing the potential for missing essential and potentially programme-enabling compounds when screening.

The advent of 384-well APCs has truly enabled APC to be considered as a frontline screening approach for ion channel drug discovery. However, as highlighted in this case study, even medium-throughput APC systems like the QPatch can be used efficiently in primary screening as well. Although using APC for screening is potentially a more expensive screen in a per well cost comparison, Chambers et al. (2016) suggested that running a single APC screen can

“Widely introduced to meet the challenges of safety screening, automated patch clamp has developed to become a core resource to support ion channel drug discovery programmes, including screening campaigns.”

significantly reduce overall programme timelines, resulting in considerable resource savings compared to fluorescent screens. These are the more obvious savings of making the pre-clinical drug discovery cascade more efficient. Still, this would clearly also add significant value to the later stages of drug discovery, clinical trials and approval for disease treatment (simply put: better data in earlier, better data out later, e.g. potential longer patent lifespans).

Concluding remarks

With the advent of APC technology data, throughput has risen dramatically, whilst the technical hurdles needed to make ion channel recordings have been lowered. This is enabling a broader range of scientific researchers, performing technically challenging ion channel recordings, with the effect of advancing the ion channel field further and faster. Arguably, the advent of planar patch-clamp and the introduction of APC are the most significant developments in ion channel recording technology since the patch-clamp revolution, in a golden decade started by Neher and Sakmann's seminal publication in 1976 (Neher & Sakmann, 1976, Hamill et al. 1981, Sakmann & Neher 1984).

Understandably, biotech, pharma and CROs were early adopters of APC technology, but more academic labs are now accessing and adopting the technology via collaborations, consortia and shared core facilities.

The TMEM16A drug discovery programme initiated by Enterprise Therapeutics is one of several emerging ion channel success stories, from discovering novel compounds, providing *in vitro* and *in vivo* proof-of-concept studies, a full drug development programme and through to clinical trials.

A key component of Enterprise Therapeutics' discovery programme was the use of APC, both as a frontline screening tool (in collaboration with work at Sophion Bioscience and Sussex Drug Discovery Centre, University of Sussex) and to support subsequent lead optimisation. The TMEM16A potentiator programme's promise has led to Roche/Genentech's acquisition, for further development towards a treatment for CF patients.

With APC technology becoming commonplace, the future

of ion channel research looks bright. This APC enabled explosion in ion channel research, and is likely to lead to significant breakthroughs in our understanding of ion channel physiology and pathophysiology.

Consequently, on the back of this accelerated research, ion channel treatments will be found helping patients across a wide range of diseases, and there is significant potential for future ion channel targeted drugs. The QPatch-driven TMEM16A potentiator discovery programme discussed here may even provide us with one such promising candidate to reach patients in the near term.

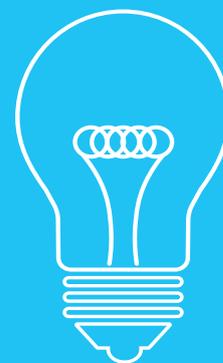
“ We are incredibly grateful for the support Sophion has provided to us and the programme. Automated electrophysiology has been **KEY** not only in finding the chemistry start points but in supporting the programme through its entire lead optimisation. The QPatch has been the primary assay and in our hands provides robust, reliable data with an exceptional correlation to the data from ion transport for TMEM16A (Ussing chamber), which is viewed by many as the gold standard assay for epithelial ion transport.

Martin Gosling
CSO Enterprise Therapeutics

”

Learnings

- Primary screening using automated patch clamp electrophysiology can be performed efficiently even with medium throughput platforms like QPatch 48.
- Primary screening on APC has many advantages over other formats with a much reduced false positive/negative rate and the ability to ask more sophisticated questions in an initial screen, e.g. state dependent modulation.
- With the ability to run unattended and on a near 24h cycle, both screening and compound profiling characterization can be rapid and efficient, saving precious development time and reducing FTE costs, and/or increase fidelity.
- Implementation of APC for primary screening can accelerate the transition to lead optimization and beyond.



References:

- Balfour-Lynn IM & King JA (2020) CFTR modulator therapies – effect on life expectancy in people with cystic fibrosis. *Paediatr Respir S1526-0542(20)30081-6*. doi: 10.1016/j.prrv.2020.05.002
- Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, Pfeffer U, Ravazzolo R, Zegarra-Moran O, Galiotta J. (2008) TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science* 322 (5901):590-4.
- Chambers, C., Witton, I., Adams, C., Marrington, L., & Kammonen, J. (2016). High-Throughput Screening of NaV1.7 Modulators Using a Giga-Seal Automated Patch Clamp Instrument. *Assay and Drug Development Technologies*, 14(2), 93–108. <https://doi.org/10.1089/adt.2016.700>
- Danahay, H. L., Lilley, S., Fox, R., Charlton, H., Sabater, J., Button, B., McCarthy, C., Collingwood, S. P., & Gosling, M. (2020). TMEM16A Potentiation: A Novel Therapeutic Approach for the Treatment of Cystic Fibrosis. *American Journal of Respiratory and Critical Care Medicine*, 201(8), 946–954. <https://doi.org/10.1164/rccm.201908-1641OC>
- Dobra, R., Edmondson, C., Hughes, D. et al. Potentiators and Correctors in Paediatric Cystic Fibrosis Patients: A Narrative Review. *Pediatr Drugs* 20, 555–566 (2018). <https://doi.org/10.1007/s40272-018-0315-z>
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch - Eur J Physiol* 391: 85–100.
- Lei, C. L., Fabbri, A., Whittaker, D. G., Clerx, M., Windley, M. J., Hill, A. P., Mirams, G. R., & de Boer, T. P. (2020). A nonlinear and time-dependent leak current in the presence of calcium fluoride patch-clamp seal enhancer. *Wellcome Open Research*, 5(152). <https://doi.org/10.12688/wellcomeopenres.15968.1>
- Li, Hongyu, Johanna J Salomon, David N Sheppard, Marcus A Mall, Luis JV Galiotta, (2017). Bypassing CFTR dysfunction in cystic fibrosis with alternative pathways for anion transport, *Current Opinion in Pharmacology*, Volume 34. Pages 91-97. ISSN 1471-4892. <https://doi.org/10.1016/j.coph.2017.10.002>.
- Mall, M. A., Luis J.V. Galiotta (2015). Targeting ion channels in cystic fibrosis, *Journal of Cystic Fibrosis*, Volume 14, Issue 5.
- Neher, E., Sakmann, B. (1976) Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* 260, 799–802. <https://doi.org/10.1038/260799a0>
- Sakmann B, Neher E (1984). Patch clamp techniques for studying ionic channels in excitable membranes. *Annu Rev Physiol* 46: 455–472.
- Sauter, D. R. P. (2016). TMEM16A on Qube 384. Sophion Application Report, 1–4.
- Schroeder BC, Cheng T, Jan YN, Jan LY. (2008) Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell* 134(6):1019-29.
- Smyth, R. L. (2020). New drug treatments for cystic fibrosis. *BMJ*, m118. doi:10.1136/bmj.m118
- Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim WS, Park SP, Lee J, Lee B, Kim BM, Raouf R, Shin YK, Oh U. (2008) TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature* 455(7217):1210-5

Authors:

Dr Martin Gosling, CSO, Enterprise Therapeutics, UK

Dr Sarah Lilley, Senior Research Fellow, Sussex Drug Discovery Centre, University of Sussex, UK

Dr Thomas Binzer, Vice President, Marketing and R&D, Sophion Bioscience, Denmark

Dr Damian Bell, Director Global Scientific Affairs, Sophion Bioscience, Denmark