New method for rapid and dynamic quantification of elastase activity on sputum neutrophils from patients with cystic fibrosis using flow cytometry

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Small molecule FRET flow cytometry is a new method that enables rapid and sensitive quantification of surface-bound elastase activity on sputum neutrophils from patients with cystic fibrosis and potentially other neutrophilic airway diseases http://bit.ly/2IegeSB

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Introduction

Increased activity of the serine protease neutrophil elastase (NE), secreted by activated neutrophils in the airways, is a key risk factor for the onset and progression of structural lung damage and lung function decline in patients with cystic fibrosis (CF) and non-CF bronchiectasis [1–6]. In addition to progressive structural lung damage, increased NE activity has been implicated in mucus hypersecretion [7–9], perpetuation of airway inflammation [10], and impaired host defence against *Pseudomonas aeruginosa* infection [6, 11–13]. These studies suggest increased NE activity in sputum or bronchoalveolar lavage fluid (BALF) as a promising biomarker of airway inflammation in CF and potentially other neutrophilic airway diseases [2–4, 6].

To date, measurements of NE activity have focused on its free activity that becomes detectable in BALF or sputum supernatant once the overall elastase burden exceeds the inhibitory capacity of endogenous anti-proteases such as α1-antitrypsin and secretory leukocyte protease inhibitor [6]. After this anti-protease shield is broken, free NE activity can be measured by various methods ranging from activity-based immunoassays [5] to fluorogenic substrate-based kinetic assays [1, 2, 5] and Förster resonance energy transfer (FRET) reporter-based assays [14–16].

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Why measure elastase activity on the surface of airway neutrophils?

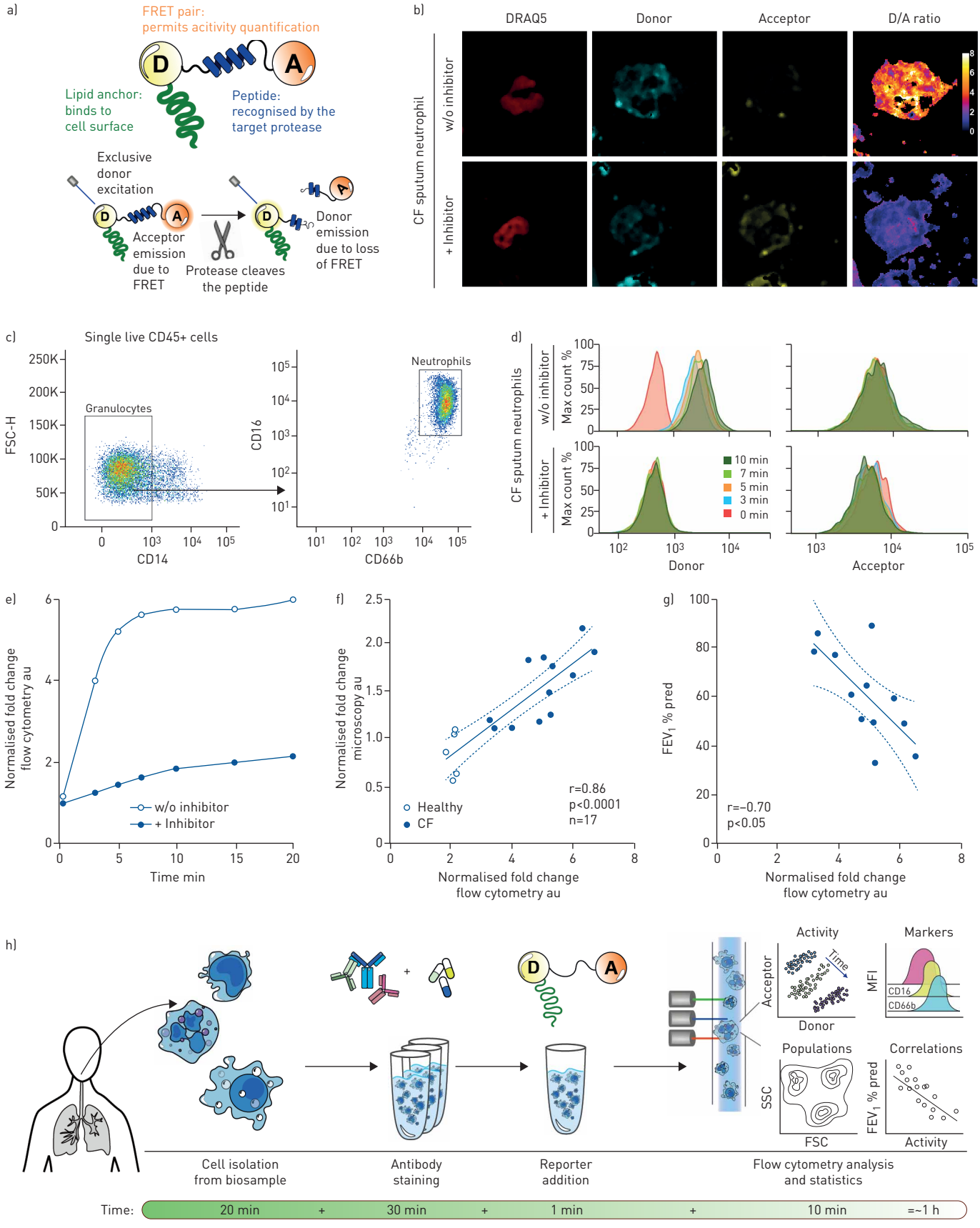
NE is a highly cationic protein resulting in binding of a substantial proportion of secreted NE to the neutrophil surface *via* electrostatic interactions [17, 18]. Previous experimental work showed that this surface-bound fraction of NE confers proteolytic activity and is resistant to inhibition by endogenous anti-proteases [17, 19, 20]. Recent observational studies demonstrated that increased exocytosis and surface-bound activity of NE are already present in young children with CF and mice with CF-like lung disease, long before free NE activity becomes detectable [14, 21]. Furthermore, it was found that surface-bound NE activity correlates with early lung damage in children and severity of lung disease in adult patients with CF independent of free NE activity [14, 22]. These results support an important role of surface-bound NE activity in pathogenesis and as a potential novel biomarker of CF lung disease across a broad spectrum of patient age and disease severity.

How can elastase activity be measured on the surface of airway neutrophils?

For measurements of surface-bound NE activity on airway neutrophils, we previously developed a lipidated ratiometric FRET reporter that inserts into the plasma membrane and contains a peptide sequence that is recognised and cleaved by NE [23, 24]. In addition, we demonstrated that this lipidated FRET reporter enables sensitive and specific measurement of surface-bound NE activity *via* change in FRET signal measured by confocal microscopy (figure 1a and b). However, widespread implementation of this method is hampered by the time required to process and prepare the samples and acquire and analyse images (the whole procedure takes up to 8 h), and by the need for expensive high-end microscopy equipment. Here, we present a new technique based on the combination of a lipidated FRET reporter and multi-colour flow cytometry, called small molecule (sm) FRET flow cytometry [25]. This technique enables quantitative assessment of NE activity on cell surfaces in a fast, robust and dynamic fashion.

How does the measurement of surface-bound elastase activity by flow cytometry work?

The lipidated FRET reporter relies on two fluorescent dyes: an energy donor (Coumarin 343) and an energy acceptor (5/6-TAMRA). The donor dye is connected to an aliphatic chain serving as a lipid anchor *via* a lysine residue and an amino acid sequence that is a substrate for NE. Two short polyethylene glycol spacers flank and connect the amino acid sequence with the acceptor and donor dyes (figure 1a) [23]. The close (<10 nm) proximity of the energy donor and acceptor allows FRET to occur efficiently when coumarin is excited. Similar to fluorophore-conjugated antibodies routinely employed in flow cytometry, this lipidated FRET reporter localises to the cell surface, where the hydrophobic moiety tethers the molecule to membrane lipids, while negative charges prevent its premature internalisation [23]. When active NE is present at the neutrophil surface, it cleaves the target peptide sequence of the FRET reporter resulting in the diffusion of the acceptor fluorophore away from the cell membrane, thereby impeding FRET (figure 1a and b). To advance FRET-based measurements of surface-bound NE activity from confocal microscopy to flow cytometry, we designed a panel of fluorochrome-conjugated antibodies consisting of anti-CD14 (monocytes, Pe/Cy7, BD Bioscience, 557 742), CD16 (neutrophils, AF700, BD Bioscience, 557 920), CD45 (leucocytes, APC-Cy7, BD Bioscience, 557 833), and CD66b (granulocytes, Pe/Dazzle594, BioLegend, 305 122) to selectively gate airway neutrophils as 7-AAD^{neg}/CD45^{pos}/CD14^{neg}/CD16^{pos} and CD66b^{pos} cells (figure 1c). In addition, we stained for CD63 (AF647, BioLegend, 353 016) as a marker for primary granule exocytosis. Spectral compensation and fluorescence minus one controls were performed for all antibodies and the live dead marker. Sputum cells were isolated as previously described [22], filtered through a 40 µm strainer to remove mucus and avoid clogging of the flow cytometer and stained with this antibody panel, as well as a viability marker (7-AAD). Cell viability in all samples was >70%. An aliquot of stained cells was incubated for 10 min at room temperature with the NE inhibitor sivelestat (CAS number 201677-61-4; Cayman Chemical, Ann Arbor, MI, USA) at a concentration of 225 µmol·L^{-1} to inhibit reporter cleavage, and these samples served as negative controls [22, 26]. Subsequently, the lipidated NE FRET reporter (NEmo-2) was added and surface-bound activity was measured by flow cytometry at the indicated time points (figure 1d and h). In our setup, the flow cytometer was equipped with three lasers emitting at 405 nm, 488 nm, and 633 nm, respectively. For the detection of protease activity, the reporter was excited with the 405 nm laser. The donor signal was detected between 425 nm and 475 nm (450/50 nm filter) and the acceptor signal between 564 nm and 606 nm (585/42 nm filter) with an upstream longpass filter (550 nm). The FRET ratio was calculated by dividing the donor and acceptor mean fluorescence intensity values measured on the gated neutrophil population at given time points. Then, the donor/acceptor ratio was normalised to the t=0 donor/acceptor ratio of the negative control to ensure intra- and inter-assay stability. A change in FRET ratio represents a semi-quantitative read-out of enzymatic activity. The optimal time point for the analysis of activity is


a)
FRET pair:
permits acitivity quantification
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A
Lipid anchor:
binds to
cell surface
Peptide:
recognised by the
target protease
Exclusive
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Acceptor
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due to
FRET
Protease cleaves
the peptide
Donor
emission
due to loss
of FRET
b)
DRAQ5
Donor
Acceptor
D/A ratio
CF sputum neutrophil
w/o inhibitor
+ Inhibitor
c)
Single live CD45+ cells
FSC-H
Granulocytes
CD14
CD16
Neutrophils
CD66b
d)
CF sputum neutrophils
w/o inhibitor
+ Inhibitor
Max count %
10 min
7 min
5 min
3 min
0 min
Donor
Acceptor
e)
Normalised fold change
flow cytometry au
w/o inhibitor
+ Inhibitor
Time min
f)
Normalised fold change
microscopy au
Healthy
CF
r=0.86
p<0.0001
n=17
Normalised fold change
flow cytometry au
g)
FEV1 % pred
r=−0.70
p<0.05
Normalised fold change
flow cytometry au
h)
Activity
Markers
Acceptor
Time
Donor
MFI
CD16
CD66b
Populations
Correlations
SSC
FSC
FEV1 % pred
Activity
Cell isolation
from biosample
Antibody
staining
Reporter
addition
Flow cytometry analysis
and statistics
Time:
20 min
+
30 min
+
1 min
+
10 min
=~1 h

FIGURE 1 Rapid quantification of elastase activity on sputum neutrophils from patients with cystic fibrosis (CF) using a lipidated small molecule (sm) FRET reporter in combination with flow cytometry. a) Structure and mechanism of action of smFRET reporters. b) Confocal microscopy images of CF sputum neutrophils showing donor/acceptor (D/A) intensities after 10 min of incubation with the surface-bound neutrophil elastase (NE) FRET reporter (NEmo-2) without (w/o) and with (+) the NE inhibitor sivelestat. c) Gating strategy to identify sputum neutrophils in smFRET flow cytometry. d) Donor and acceptor mean fluorescence intensities of NEmo-2 measured in the absence (upper panels) and presence (lower panels) of NE inhibitor by flow cytometry on ~1000 sputum neutrophils of a patient with CF. e) Donor/acceptor ratio of NEmo-2 determined by flow cytometry over a period of 20 min. Mean fluorescence intensities of donor and acceptor were derived from ~1000 CF sputum neutrophils in the absence and presence of the NE inhibitor. Donor/acceptor ratio was normalised to the inhibited negative control donor/acceptor ratio at t=0 min. f) Correlation between flow cytometry and confocal microscopy measurements of surface-bound NE activity at t=10 min on sputum neutrophils from healthy individuals and patients with CF. g) Correlation between surface-bound NE activity measured by smFRET flow cytometry and forced expiratory volume in 1 s (FEV_1) % predicted in patients with CF. Correlation analyses were performed using the Spearman rank order method. A p-value <0.05 was considered statistically significant. h) Experimental workflow of smFRET flow cytometry. Sputum cells are isolated from healthy and diseased subjects. Cells are stained with an adequate antibody panel that allows the identification of the cell population of interest. After antibody staining, the reporter is added to the cells, the sample is subsequently analysed by flow cytometry, surface-bound protease activity is determined, and results are available within approximately 1 h.

within the plateau phase of the donor/acceptor ratio which we identified for our experimental conditions to be at t=10 min (figure 1e).

To validate this new method, we analysed surface-bound NE activity with NEmo-2 on sputum neutrophils from 12 patients with CF (mean±SD age 30.9±11.8 years, range 22.1–58.2 years) and five healthy controls (mean±SD age 36.6±10.0 years, range 27.1–49.1 years). CF patients had the following *CFTR* genotypes: F508del/F508del (n=6), F508del/I507del (n=1), F508del/CFTR dele 17 (2.5 kb) (n=1), F508del/T1299I (n=1), R553X/M1101K (n=1), W1282X/2991del32 (n=1), and G542X/X (n=1). Seven patients were chronically colonised with *Pseudomonas aeruginosa*, eight with *Staphylococcus aureus* and three with *Aspergillus fumigatus*. At the time of sputum collection, 10 patients were clinically stable and on inhaled or oral antibiotics as standard of care, and two patients had a pulmonary exacerbation and were treated with *i.v.* antibiotics. We performed side-by-side measurements using confocal microscopy and the new smFRET flow cytometry on sputum neutrophils from all 17 subjects. All analyses either performed by confocal microscopy or flow cytometry were based on single cell measurements and data are reported as mean of all cells measured from the same individual (CF patient or healthy control). We found a strong correlation between surface-bound NE activity determined by confocal microscopy and flow cytometry (figure 1f). In addition, these analyses indicated that smFRET flow cytometry detects surface-bound NE activity with less variability and a larger dynamic range (fig. 1e and f). Further, in line with previous results obtained by confocal microscopy [22], forced expiratory volume in 1 s % predicted of patients with CF correlated inversely with surface-bound NE activity measured by smFRET flow cytometry (figure 1g).

Taken together, smFRET flow cytometry substantiates previous results obtained by confocal microscopy demonstrating increased surface-bound NE activity on neutrophils sampled from CF airways. In addition, smFRET flow cytometry has several advantages over confocal microscopy. First, smFRET flow cytometry can discriminate between live and dead cells and measurements of NE activity are only based on live sputum neutrophils, thereby overcoming difficulties previously encountered in measurements by confocal microscopy, such as autofluorescence of epithelial cells and interference by extracellular DNA, mucus or dying cells that are excluded from the measurement by flow cytometry. Second, a much larger number of neutrophils can be analysed. Compared to confocal microscopy, where single cell analyses were limited to 100–200 cells, flow cytometry enabled us to increase cell numbers to ~1000 neutrophils per measurement. This cell number yielded an improved signal-to-noise ratio, but still allowed us to perform paired measurements in sivelestat-treated controls from the same sample, as well as measurements in comparable numbers of neutrophils from CF patients and healthy controls containing substantially lower neutrophil counts. Third, smFRET flow cytometry enables assessment of dynamic changes in surface-bound NE activity over time. Finally, the experimental procedure and data analysis for measurements of NE activity by smFRET flow cytometry is vastly simplified and substantially less time-consuming compared to the confocal microscopy method. In fact, we were able to perform standardised NE activity measurements on neutrophils by flow cytometry within approximately 1 h (figure 1h), which would be a suitable time for performing this test in the clinical diagnostic setting.

How can this new method for quantitative assessment of elastase activity on sputum neutrophils be used in the future?

This new method of smFRET flow cytometry enables direct, rapid and dynamic measurement of NE activity on the surface of airway neutrophils (figure 1h). The method can be applied to quantify surface-bound NE activity on neutrophils isolated from sputum of patients with CF and potentially other neutrophilic airway diseases such as bronchiectasis [5]. In addition, data from previous studies in mice with CF-like lung disease using the NEmo-2 reporter and confocal microscopy support the idea that

measurements can also be performed on cells isolated from BALF [21]. In the clinical arena, we expect smFRET flow cytometry to be used as a versatile translational research tool for more comprehensive studies of the relationship between membrane-bound NE activity and lung disease severity and progression, and studies of its specific value as a biomarker compared to free NE activity, especially at early stages of airway inflammation. In addition, smFRET flow cytometry may also be used as a powerful tool to determine response to therapy to a range of interventions at the level of neutrophil activation and NE secretion. Such interventions may include endogenous and small molecular weight NE inhibitors as a targeted anti-inflammatory strategy [6], as well as emerging highly effective CFTR-directed therapeutics targeting the underlying molecular defect in patients with CF [27, 28]. Furthermore, in combination with cell-specific markers, we envision smFRET flow cytometry as a powerful basic research tool for mechanistic studies of neutrophil pathobiology in the lung, blood and other organs. At a broader scale, this method may also be applied to studies of other proteases on neutrophils and/or other cell types. Beyond NE, lipidated FRET reporters have already been generated for cathepsin G and macrophage elastase (matrix metalloproteinase 12) and are under development for a spectrum of other proteases that have been implicated in CF and other chronic lung diseases [25, 29–31]. With the emergence of these additional reporters, smFRET flow cytometry will also provide new possibilities to obtain a more comprehensive understanding of the dysregulation of protease networks in disease pathogenesis, which may lead to the development of more sensitive biomarkers and novel therapeutic strategies to restore protease–antiprotease balance in CF and other chronic inflammatory lung diseases.

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