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**ARTICLE**

# A research-led flexible cell biology practical for biological sciences undergraduate and postgraduate degree courses

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**Abstract**

A challenge in the pandemic era is to implement effective but flexible practical teaching for biological sciences courses. Such teaching needs to deliver conceptual, analytical and practical skills training while having the option to rapidly respond to health and safety issues, local regulations, staff and student concerns. In this paper, we describe a set of cell biology practicals (mini-project) that meets many of these requirements and provides flexibility in providing skills training both through online and in practical laboratory environments. We have used a human adenocarcinoma cell line A431 stably transfected with a fluorescent cell cycle reporter as a biological model to deliver training through discrete work packages encompassing cell culture, fluorescence microscopy, biochemistry and statistics. How such work packages can be modified to, an online format either partially or completely is also described. Furthermore, the activities can be adapted for teaching both undergraduate and postgraduate level courses to ensure effective skills training which is applicable to a wide range of biological degree programs and levels of study.

**KEYWORDS**

A431 cancer cell line, biochemistry, cell biology, cell culture, fluorescent reporter, microscopy, practical, statistics

**1 | INTRODUCTION**

The teaching of practical and quantitative skills in modern biological sciences programs can be challenging, given the large increase in methodologies, databases and

biological systems under study. Thus, development of flexible practical programs that can deliver training in core techniques, quantitative and broader transferable skills and can be rapidly adapted for use in different degree programs and changing circumstances is particularly valuable. This is especially true in the pandemic era, where issues such as social distancing, health and safety, local regulations, staff and student concerns need to be considered carefully.

Here we describe a set of research-led, modern, flexible cell biology practicals, which can not only deliver

**Abbreviations:** EGFR, epidermal growth factor receptor; ERK1/2, extracellular-regulated kinase 1 and 2; FUCCI, Fluorescent ubiquitinated cell cycle indicator; mAG, monomeric Azami Green; MAPK, mitogen-activated protein kinase; mKO, monomeric Kusabira Orange; p38 MAPK, 38 kDa mitogen-activated protein kinase.

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essential skills but also motivate and excite students about studying biological systems. These practicals have been developed with the following goals for student learning (i) to provide training in wet laboratory work in key cell biology techniques used in modern research and their application to ideas and concepts at the forefront of science; (ii) provide experience of how research is carried out through a series of linked experiments, integrating research planning, experimentation, analysis, and trouble-shooting; (iii) enhance students ability to work with mathematics and statistics applied to biological datasets; and (iv) support students in their development of collaborative and team work capabilities.

Our considerations as part of the design phase were (i) the selection of a biological model that is easy to use in both small and large group teaching settings, (ii) the model is compatible with delivering a range of practical and conceptual skills relevant to a range of biological science degree programs, (iii) the degree of technical complexity is amenable to modification depending on the training level (e.g., undergraduate or postgraduate programs), and (iv) technical support, equipment and resources are in place to support academic delivery and ensure a high-quality student learning experience.

To achieve these objectives, the biological model selected for use is an A431 human cancer cell line stably expressing a fluorescent cell cycle reporter (A431-FUCCI) to train students at the bench and *in silico*. In addition to developing technical expertise, we used this A431-FUCCI cell line to develop student's theoretical knowledge about cancer, signaling, cell cycle, and targeted drug therapy. Students carried out practical work in discrete work packages, culminating in a written output in the form of a scientific research paper. Below, we provide an overview of each of the work packages and indicate how the practicals can be rapidly adapted to an online format if required.

## 2 | CELL BIOLOGY COURSE STRUCTURE AND OVERVIEW

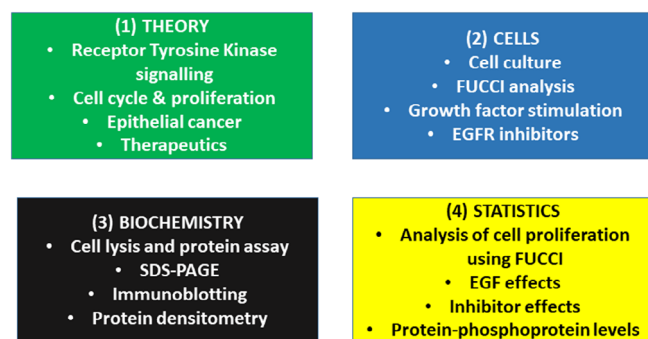
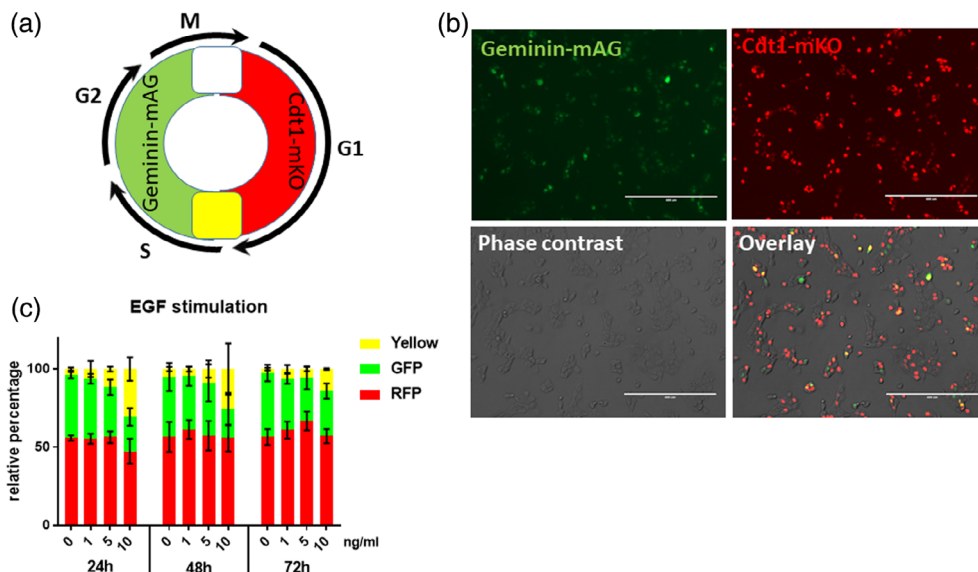
The course described here was undertaken by students' mid-way through a 3-year or 4-year undergraduate degree, registered on a range of degree areas: molecular and cell biology, biochemistry, genetics, and medical sciences. Class size can range from 50 to 80 students. The course runs over a period of 8 weeks, with students spending approximately 6 h per week on the practical and associated computational/analytical activities. This duration can be adapted depending on the level of skills training required by the degree program. Students typically work in pairs within larger teams, with the teams collaborating to share activities and data sets.

This mini-project comprises a set of linked practicals utilizing the A431 cancer cell line transfected with fluorescent cell cycle reporter proteins FUCCI (fluorescent ubiquitinated cell cycle indicator). Miyawaki and colleagues pioneered the development and use of new fluorescent proteins for research applications.<sup>1</sup> The cloning and characterization of these new proteins led to the development of the FUCCI.<sup>2</sup> Here, two different fluorescent proteins, monomeric Azami Green (mAG) and monomeric Kusabira Orange (mKO), when expressed in cells and whole animals can detect dividing cells. Furthermore, this dual fluorescent reporter can show the stages of a cell cycle depending on the color of the images when simultaneously overlaid using a live cell microscopy imaging system. Cells are in G1 (red), G2 (green) or S phase (yellow) depending on the presence or absence of the mAG or mKO reporter molecules, which have been tagged with specific sequences that either mediate degradation or stabilization in particular phases of the eukaryote cell cycle (Figure 1a).

We have cloned this FUCCI reporter into a lentiviral plasmid and generated a recombinant lentivirus that was used to transduce A431 adenocarcinoma cancer cells. After selection with puromycin, stable A431-FUCCI clones were isolated, propagated in puromycin media and analyzed by microscopy. A431-FUCCI cells routinely displayed an expression of mAG (green) and red (mKO) staining patterns. Most cells within the A431-FUCCI population exhibited red staining indicative of the G1 phase of the cell cycle corresponding to the expression of mKO fusion protein (Figure 1b). A smaller proportion of the cell population showed green staining indicative of G2 phase of the cell cycle corresponding to expression of mAG fusion protein (Figure 1b). These findings show that this is indeed a functional FUCCI-based system, which could be used to monitor A431 cancer cell proliferation. In response to EGF stimulation, there are changes in the proportion of red, green, and yellow cells consistent with increased cell proliferation over time (Figure 1c).

Our course is delivered through a series of linked practical work packages (WPKG #1–4) that are schematically depicted in Figure 2. These are structured to progress students from training in theoretical concepts to practical applications including mathematical and statistical training in data analysis and ending in synoptic assessment of their learning. The hands-on practical training is complemented with digital resources (e.g., screen casts or videos presenting how to use a particular software or analyze data). Sessions are delivered by academic staff (lecturers) supported by doctoral students as teaching assistants. During the weekly classes, there are substantial opportunities for students to engage in discussions with the lecturers and with the teaching assistants (demonstrators) about their ongoing data

**FIGURE 1** Expression of the FUCCI reporter in mammalian cells. (a) Schematic showing expression of the FUCCI reporter during the human cell cycle. (b) Microscopy images of living A431-FUCCI cells showing expression of Geminin-mAG (green), Cdt1-mKO (red), phase contrast image of cells, and composite overlay of all images. Bar, 400 nm. (c) Quantification of FUCCI reporter expression in response to stimuli 0–10 ng/mL EGF over 24–72 h.



**FIGURE 2** Schematic of four (numbered 1–4) linked work packages to deliver cell biology skills training.

collection and analysis. The discussions are structured through the completion of worksheets, either pre-practical or post-practical, dependent on the stage of the practical project. These worksheets are designed to support experimental planning, data analysis, and interpretation. Dedicated technical support is provided by a teaching technician. While the classes are delivered in well-equipped teaching laboratories, students are able to access research facilities for some of the work (e.g., imaging suite for fluorescence microscopy).

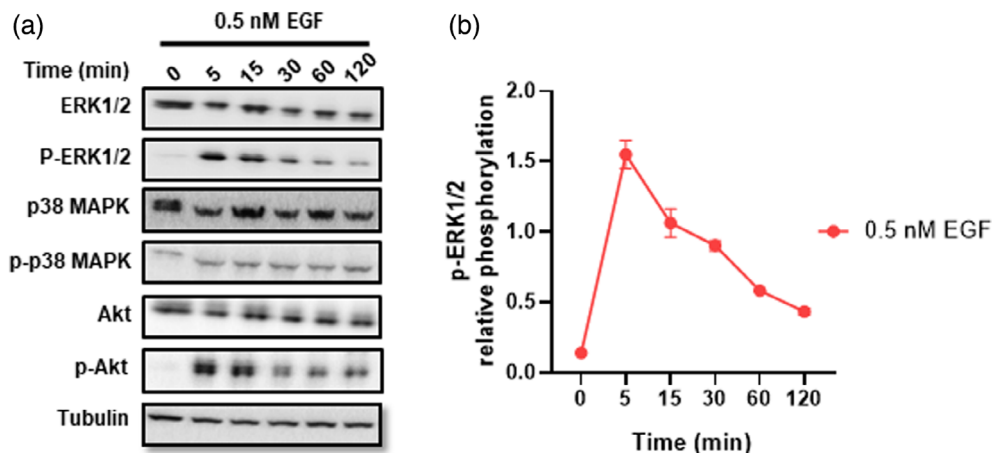
## 2.1 | Work package 1: Understanding the theory

As the first step, students are tasked to actively engage with published literature to explore and build their understanding of theoretical concepts underpinning the project work they are about to embark on (Figure 2). Students are provided with a set of review articles<sup>2,3</sup> on signal transduction, cancer biology, and applications of

FUCCI. They are asked to explore how epidermal growth factor (EGF) regulated signal transduction works and how this may go wrong in epithelial cancers. This also raises the concept of using pharmacology to target EGF receptor (EGFR/ErbB1) signal transduction in specific cancer states. Students allocated to teams, work together to deliver a short presentation (e.g., 5 min presentations) to the rest of the class followed by questions from the audience comprising members of their class, lecturers and teaching assistants. Members within the student team research and present on different dimensions of the project and in this way peer-teach. Collaboration with other group members is via discussion boards set up on the University's virtual learning system or other tools students may choose to use. Depending on the level of degree program, this task can be increased in length with longer presentations and requirement for more references and reading around the topic.

## 2.2 | Work package 2: Cell culture, fluorescence microscopy, and data collection

The second work package (WPKG #2) delivers practical training in cell culture, fluorescence microscopy, and data collection (Figure 2). First, the students are trained in simple cell culture techniques, including splitting and counting cells and sterile culture techniques. This includes hands-on training combined with online tutorials developed either in-house (e.g., short videos) or commercially available (e.g., Labster). Students then learn how to acquire light and fluorescence microscopy images using a digital microscopy platform to evaluate



**FIGURE 3** Immunoblotting of EGF-stimulated A431-FUCCI cells. (a) Epidermoid carcinoma A431-FUCCI cells were stimulated with 0.5 nM EGF for 5, 15, 30, 60, and 120 min lysed and processed for immunoblot analysis of signal transduction native or phosphorylated (p-ERK1/2, p-p38 MAPK, p-Akt) proteins. (b) Quantification of relative p-ERK1/2 levels upon 0.5 nM EGF stimulation.

cancer cell growth kinetics (Figure 1b,c). Finally, they learn how to collect and analyze the data using freeware programs such as NIH Image J (<https://imagej.nih.gov/ij/>).

Students are provided with cells in 6, 12, or 24-well plates at 10%–75% confluency depending on the treatments. Such treatments include dose titration of EGF (0–50  $\mu\text{g}/\text{mL}$ ) treated over 0–72 h. Furthermore, EGFR tyrosine kinase inhibitors (e.g., erlotinib) can be titrated (0–10  $\mu\text{M}$ ) in the presence or absence of EGF to assess effects on cell proliferation (WPKG #2), signaling and phosphorylation (WPKG #3). Treated cells are usually analyzed every 20–24 h, and each well is sampled three times at least. For quantification, the field of view is best analyzed using a 10 $\times$  or 20 $\times$  dry objective lens, this ensures that 20–50 cells are sampled in each field. For statistical significance, each experiment is carried out in triplicate or more if needed. The captured data is then analyzed as described in work package 4 below. The cell imaging data can be shared between groups to increase the size of the data set, allowing students to manipulate larger data sets. Sharing of data sets also allows a greater variety of growth conditions to be assessed, extending the breadth and depth of analysis and, therefore, the intellectual and technical challenge of the activities.

### 2.3 | Work package 3: Biochemical analysis, signaling and quantification

In the third work package (WPKG #3) students are trained in biochemistry, signaling and quantification (Figure 3). First, A431-FUCCI cells are grown and treated with EGF (*with and without* inhibitor) in 6-well format and lysed in 200–300  $\mu\text{L}$  of lysis buffer. Then the students assess the protein concentration of the cell lysates using a

BCA assay within a 96-well plate format and calculate the amount of total cellular protein (10–20  $\mu\text{g}$ ) that is needed to be loaded per lane during SDS-PAGE. They then load their cell lysates and protein marker on pre-cast SDS-PAGE gels and use electroblotting to transfer the proteins onto nitrocellulose membranes for immunoblot analysis.

Typically, for each test protein, for example, a protein kinase is compared to its activated and phosphorylated version, e.g. phospho-ERK1/2 (Figure 3a). Each student or student pair run 2 gels, which are blotted onto nitrocellulose and then cut into upper (60–200 kDa) and lower (60–10 kDa) blots for probing for different signaling proteins depending on the molecular mass of each protein. For example, EGFR (180 kDa), ERK1/2 (42/44 kDa doublet), p38 MAPK (38 kDa), and AKT (56 kDa). Loading controls actin (40 kDa) and tubulin (50 kDa) are probed separately (Figure 3a). Immunoblot analysis is based on HRP-conjugated secondary antibodies and enhanced chemiluminescence (ECL) detection using a digital imaging workstation set to collect exposures varying from 30 s to 10 min depending on antibody cross-reactivity. It is important to note that ECL detection is usually done by an experienced teaching assistant or a student(s) closely supervised by teaching assistants. Due to instrument availability, usually 1–2 teaching assistants process each individual blot for the ECL reaction and collect digital datasets, which are sent to each student(s).

It is important for students to understand the link between antigen levels, detection, variation and quantification. Thus, students are asked to quantify the density of each band on the blot using computational software such as NIH Image J and to calculate relative protein and phosphoprotein levels within each experiment. One useful equation to support the analysis is as follows, depicted as a histogram in Figure 3b.

Native protein level = Pixel intensity of native protein band/Pixel intensity of tubulin control band.

Phosphoprotein level = Pixel intensity of phosphoprotein band/[Pixel intensity of native protein band  
× Pixel intensity of tubulin control band]

Typically, due to time constraints, it is difficult for one student or a student pair to probe all the signaling proteins of interest shown in Figure 3a. Thus, students collaborate in their teams to allocate different signaling proteins to probe and then share the data for a complete picture of signaling pathways and impact on these with and without drug treatment to be built up.

## 2.4 | Work package 4: Data analysis and interpretation

To support the analysis of the captured data, students are provided with a series of short online-tutorials (screen-casts) showing how to count the fluorescent cells using computational software, transport it to a program such as Excel or OriginPro and statistically analyze the data. The final part is WPKG #4 (Figure 2) which is training in data analysis using different software packages. Wherever possible, freeware such as NIH Image J (<https://imagej.nih.gov/ij/>) and R Studio is used for data analysis. However, R Studio use requires substantial prior training for effective implementation. Programs such as OriginPro (<https://www.originlab.com>) or GraphPad (<https://www.graphpad.com/>) which are easy to use but require site licenses can also be utilized. The aim here is to train students in quantifying visual data (microscopy images, western blots) and undertaking statistical analyses so that meaningful conclusions can be drawn. The key learning point to impart is the use of different software packages to analyze numerical datasets to generate graphs or histograms, which enables comparisons of baseline ( $t = 0$ , nontreated) to different treatments (e.g., EGF time course). Furthermore, the students are expected to assess biological effects numerically in % change or fold change, to ensure that scientific conclusions from practical work are based on numerical values.

Such work can be done flexibly, either online or in-person, in small or large classes. Usually, such classes are scheduled in groups with a specific teaching assistant assigned to a group of 8–15 students. Here, the students bring their raw data and are asked to process the data using the different software packages provided by the

institution. In addition, students are asked to link the finding from the earlier cell culture experiments (WPKG #2) with the later biochemical experiments (WPKG #3) drawing on their theoretical understanding (WPKG #1). Students are “challenged” to explore key questions such as how cell cycle kinetics change with different conditions (e.g., growth factor concentration or drug treatment), how this links to the activity of the cell signaling proteins, what are the cellular outcomes (e.g., proliferation, cell death), and how does this inform strategies for treating cancers?

## 3 | ADAPTING THE WORK PACKAGES FOR ONLINE DELIVERY

One advantage of this practical is that it can be rapidly adapted to an in silico format to deliver essential skills training. Although it is not ideal for students not to experience the practicality and enjoyment of observation of cells and carrying out biochemical experiments, the pandemic era has forced safety considerations to override teaching directives. During the pandemic when no face-to-face teaching was possible, we adapted all four work packages to an online format (Figure 4). WPKG #1 and #4 can be delivered online relatively easily using virtual

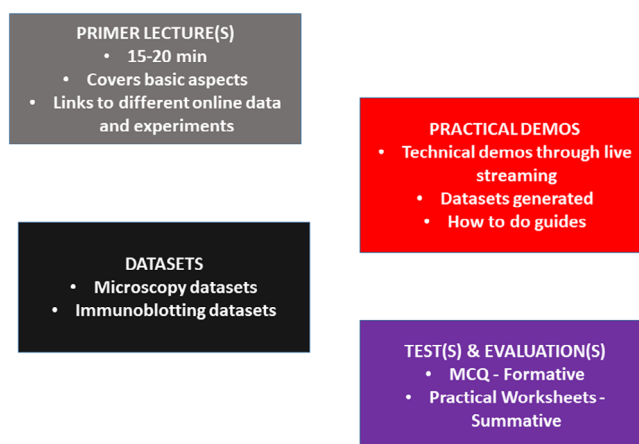


FIGURE 4 Structure and delivery of an online module.

platforms that support synchronous activity and interactive group work.

WKPG #2 and #3 requires wet lab work and here we used live streaming of practical sessions supplemented with exemplar data sets for students to analyze. Live streaming was carried out by academics with technical support and allowed students to not only view the practicals but also engage in discussions.

#### 4 | FINAL COMMENTS

Our aim is to deliver effective practical skills training in investigating and understanding biological systems. Our biological system of choice is a cancer cell model expressing a fluorescent reporter linked to the cell cycle and cell proliferation. First, we established a firm basis of theoretical knowledge of cellular signaling in response to soluble factors, a conserved phenomenon across the eukaryote kingdom. Second, we developed the thinking toward the application of such understanding to cancer therapy. Third, the development of cell-based and biochemical skills allied with data collection. As described, it is also feasible to provide such previously collected datasets allied to online training videos and lectures depending on a number of factors in the pandemic era. Finally, the development of mathematical and statistical skills is an essential feature. This can be easily adapted to different levels of undergraduate and postgraduate training to enable students to be presented with challenging tasks appropriate to their skills levels.

The advantages of adopting a flexible practical with a suite of skills that can be adapted to both face-to-face and online formats are essential in the current era. With the waning and waxing of the SARS-CoV-2 pandemic in different countries worldwide, this is further complicated by how local regulations affect practical teaching of biological degrees in higher institutions. By providing a detailed breakdown of the thinking behind the development and implementation of this practical, we hope to provide a useful skills package, which could be implemented anywhere. Furthermore, there is substantial flexibility in the adaptation of similar or related biological systems using the same linear training progression in theory, practical, mathematical, and statistical training skills.

What are potential disadvantages or handicaps to this practical? One consideration is the substantial investment in equipment and personnel. The digital imaging systems for microscopy and immunoblotting require investment; originally, research-based laboratories provided such systems for use until it was established that these were good systems for teaching use. Furthermore, using such

systems purely for teaching is not cost-effective, so such systems have dual use for both teaching and research as required. The costs of the practical work, including cell culture medium, growth factor(s), inhibitors, antibodies, pre-cast SDS-PAGE gels, rigs, and electroblotting equipment, is not inconsiderable. Finally, experienced academics, graduate teaching assistants, and technicians to support the practical are essential, and prior training is needed to ensure smooth running.

Nonetheless, we feel that this practical is an excellent basis for delivering cutting-edge skills training for a variety of biological sciences degree programs. In our own institution, a number of different Bachelor of Science (BSc) level degree courses have adopted this including Biological Sciences, Biomedical Sciences, Biochemistry, Biotechnology, Biology and Natural Sciences. We have run this practical both in face-to-face and in silico formats and the students have been able to achieve the associated learning outcomes. The student feedback has generally been highly positive—some highlights are presented below:

81% of respondents agreed or strongly agreed that the practicals helped them to understand the importance of quantifying visual data sets and that the practicals had improved their technical capabilities in cell culture techniques and live cell microscopy imaging in particular.

100% of the respondents agreed or strongly agreed that the resources provided to support learning were valuable (e.g., reading, statistics e-package).

Imaging of live cells using Evos microscopes was identified most frequently by students as the aspect they enjoyed the most.

Additional support with the analysis of data sets was identified as an area where further training would be useful.

This fluid practical format allowed us to rapidly incorporate suggestions by students, staff, technicians and teaching assistants, and evolve the skills teaching as we progress. The FUCCI system is a tremendous and useful tool for biological sciences teaching. Miyawaki and colleagues have provided a better FUCCI-2 system where additional cell cycle states can be monitored.<sup>3</sup> In the future, the application of FUCCI-based systems to whole animals (flies, worms, zebrafish, etc.) and plants could

support practical skills teaching in neuroscience and ecology as well. Again, this requires an investment in time and effort: probably 6–12 months of developing systems from scratch, or collecting the systems, equipment, tools, and reagents to run tests of these practicals before implementation in groups or classes. Our practical handbook is freely available upon request, and we only request that our work be referenced appropriately in this context.

## 5 | MATERIALS AND METHODS

### 5.1 | Reagents

All cell culture reagents were from ThermoFisher (Loughborough, UK). Epidermal growth factor was from PeProtech (London, UK).

### 5.2 | Antibodies

EGFR, p-EGFR, tubulin, actin, ERK1/2, p-ERK1/2, p38 MAPK, p-p38 MAPK, Akt, and p-Akt.

### 5.3 | Cell culture

The human A431 adenocarcinoma cell line (American Tissue Culture Collection, CRL-1555) was grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1 mM non-essential amino acids, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin on tissue culture grade T75, 6-well, and 24-well plates (Fisher Scientific, Loughborough, UK). For convenience during large-scale teaching, only DMEM containing FBS and penicillin–streptomycin was used. For starvation of cells or growth in non-serum formulation, DMEM alone (with antibiotics) was used. A431 cells were transduced with a lentivirus carrying the FUCCI reporter with a puromycin resistance marker for selection. After transduction, cells were selected in complete DMEM with 1 µg/mL puromycin. Cells were routinely passaged in media with puromycin, but this was not necessary when the cells were used in practical teaching, only DMEM containing FBS and penicillin–streptomycin was used. After removal of medium, cells were fixed by incubation in neutral buffered 10% formalin (Merck) that was stored at room temperature. Once fixed, cells could be stored at 4°C (refrigerator or cold room) in the dark, and viewed over 1–2 weeks without any detectable change in fluorescence.

### 5.4 | Microscopy

Cells were grown to sub-confluence (10%–75%) depending on the experimental protocol. Living or fixed cells were directly viewed in situ using an automated microscopy workstation EVOS FL Auto 2 running on a Windows platform (ThermoFisher). The microscope was equipped with DAPI (Ex 340 nm/Em 460 nm), GFP (Ex 488 nm/Em 510 nm), and RFP (Ex 532 nm/Em 588 nm) filtersets for viewing DAPI dye bound to nuclear DNA, mAG, and mKO fluorescent proteins respectively. Images were captured at 24 and 48 h. For each field, four images were taken using different filters: phase contrast (transmitted light), GFP, RFP, and overlay (GFP + RFP + phase contrast). The cells were counted manually using NIH Image J software. All analyzed data were expressed as mean  $\pm$  standard error. The statistical significance of differences between different experimental groups was determined using a *t*-test and *p* values < 0.05 were considered significant.

### 5.5 | Biochemistry

Cells were grown to sub-confluence (50%–75%), rinsed twice in ice-cold PBS using aspiration and lysed at room temperature in 2% (w/v) SDS, PBS pH 7.4, 1 mM PMSF with range of volumes from 0.2 to 2 mL. Aliquots of each cell lysate (0, 2, 5, and 10 µL) were assayed in duplicate compared to a BSA (0, 2, 4, 8, and 10 µg) standard curve (also in duplicate) using a 96-well plate spectrophotometer preset for BCA analysis using BCA assay kit (ThermoFisher). Cell lysates (10 or 20 µg) was electrophoresed alongside prestained AccuMarQ protein markers covering 10–245 kDa (Badrilla, Leeds, UK) using precast SDS-PAGE gels (Bio-Rad Laboratories). Proteins were then transferred to nitrocellulose membranes using the Bio-Rad Trans Blot Turbo Transfer System.

Nitrocellulose membranes were incubated in blocking buffer [1 mg/mL BSA, TBS pH 7.4, 0.1% (w/v) Tween-20] for 30–60 min at room temperature with gentle shaking. Blots were then incubated with 0.1 µg/mL primary antibody in blocking buffer (1 mg/mL BSA, TBS pH 7.4, 0.1% (w/v) Tween-20) overnight for 16–20 h with gentle shaking at 4°C (cold room). Note that it is essential that when using anti-phospho antibodies, all incubations must be done in TBS-T [TBS pH 7.4, 0.1% (w/v) Tween-20] only. Incubation in phosphate-based buffers will block phospho-specific antibody binding to target antigens. Blots were rinsed extensively in TBS-T for 15 min, then incubated with HRP-conjugated secondary antibody at 1:2000 or 0.1 µg/mL (1 mg/mL BSA, TBS-T) where appropriate for 1–2 h at room temperature. Blots were rinsed

extensively in TBS-T for 15 min, then kept in TBS until ready for imaging. Blots were exposed to ECL substrate as per manufacturer's instructions (Thermo Fisher Scientific) and visualized using a G:Box Chemi chemiluminescence imaging workstation (Syngene, Cambridge, UK). Different exposures (30 s to 10 min) were used to ensure that all data was captured. Normal light images (pre-stained protein markers) were collected and overlaid onto each ECL image to check for the position of antibody-reactive bands. Band signal intensity was quantified using software (Syngene) using pixel intensity on a 0 to 4096 greyscale. Each phosphoprotein was normalized relative to its non-phosphorylated protein and loading control and presented graphically to show change in activity under different treatment conditions.

## 5.6 | Student feedback

Student feedback was gathered through the administration of an online survey post-completion of the practical classes. Questions included a mix of closed and open questions relating to students' experiences of their laboratory work, online resources, supporting material, and effectiveness of training.

### AUTHOR CONTRIBUTIONS

**Sreenivasan Ponnambalam, Aysha Divan:** Conceptualization and methodology; **Areej Alzahrani, Faheem Shaik, Joanna Mitchell:** validation; **Sreenivasan Ponnambalam, Aysha Divan, Michael A. Harrison:** formal analysis; **Sreenivasan Ponnambalam, Aysha Divan:** Conceptualization and methodology; **Areej Alzahrani, Faheem Shaik, Joanna Mitchell:** validation; **Sreenivasan Ponnambalam, Aysha Divan, Michael A. Harrison:** formal analysis; **Sreenivasan Ponnambalam:** resources; **Sreenivasan Ponnambalam, Aysha Divan, Michael A. Harrison:** data curation; **Sreenivasan Ponnambalam, Aysha Divan, Michael A. Harrison:** writing – review and editing; **Sreenivasan Ponnambalam, Aysha Divan:** visualization; **Sreenivasan Ponnambalam, Aysha Divan:** supervision; **Sreenivasan Ponnambalam, Aysha**

**Divan:** project administration; **Sreenivasan Ponnambalam:** funding acquisition. All authors have read and agreed to the published version of the manuscript.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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