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Measurement of constitutive MAPK and PI3K/AKT signaling activity in human cancer cell lines

Kim H.T. Paraiso^{1,2}, Kaisa Van Der Kooi³, Jane L. Messina³, and Keiran S. M. Smalley^{1,2,*}

¹Department of Molecular Oncology, The Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL, 33612

²Department of Cutaneous Oncology, The Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL, 33612

³Department of Pathology and Cell Biology, University of South Florida College of Medicine, 12901 Bruce B. Downs Boulevard, MDC 11, Tampa, FL 33612

Abstract

The growth and survival of cancer cells is often driven by constitutive activity in the mitogen activated protein kinase (MAPK) and phospho-inositide 3-kinase (PI3K)/AKT signaling pathways. Activity in these signal transduction cascades is known to contribute to the uncontrolled growth and resistance to apoptosis that characterizes tumor progression. There is now a great deal of interest in therapeutically targeting these pathways in cancer using small molecule inhibitors. In this chapter we describe methods to measure constitutive MAPK and AKT activity in melanoma cell lines, with a focus upon Western blotting, phospho-flow cytometry and immunofluorescence staining techniques.

Keywords

melanoma; MAPK; ERK; AKT; PLX4032; PLX4720; therapy

1. INTRODUCTION

Tumor cells are characterized by an escape from physiological mechanisms of growth control and cell survival. These processes can be driven through multiple mechanisms including the acquisition of activating mutations in receptor tyrosine kinases (RTKs) (such as *Her2/Neu* in breast cancer and *Bcr-Abl* in chronic myeloid leukemia), mutations in oncogenes (including *KRAS* in lung cancer and *BRAF* in melanoma), loss of expression/ mutations in tumor suppressors (such as *PTEN* and *p53*) and autocrine growth factor loops (Cully and Downward, 2008; Salmena et al., 2008; Smalley, 2003; Wong et al.). Although diverse, these driving oncogenic events often rely upon the activation of a common set of signal transduction pathways to mediate their effects upon tumor behavior. The most highly studied intracellular signaling cascades in the context of cancer are the mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways.

*To whom correspondence should be addressed: Tel: 813-745-8725, Fax: 813-745-4384, keiran.smalley@moffitt.org.

Under physiological conditions, the MAPK pathway is primarily responsible for transducing extracellular growth signals, generated through the interaction of ligands with their respective RTKs, to the interior of the cell via the activation of the Ras-family GTPases. In its GTP-bound state, Ras activates a number of downstream signaling cascades involved in controlling cell growth and behavior (Cully and Downward, 2008). One such Ras-activated pathway is a family of protein serine/threonine protein kinases known as the MAPK cascade (Robinson and Cobb, 1997). Initially, Ras interacts with and activates the serine/threonine protein kinase Raf, which exists in 3 isoforms: ARAF, BRAF and CRAF (Stokoe et al., 1994; Wellbrock et al., 2004). Once active, Raf serine phosphorylates MEK1 and MEK2, (Crews et al., 1992; Dent et al., 1992) which in turn tyrosine/threonine phosphorylates extracellular-signal regulated kinase (ERK) 1 and ERK2 (Kyriakis et al., 1992). Upon activation, the ERKs either phosphorylate cytoplasmic targets or migrate to the nucleus (Lenormand et al., 1993) where they phosphorylate and activate a number of transcription factors such as c-Fos and Elk-1 (Treisman, 1994).

The aberrant activation of the MAPK pathway is implicated in the growth and pathological behavior of many cancer types. In melanoma, constitutive MAPK signaling arises through activating mutations in *NRAS* (15–20% of cases), *BRAF* (50% of cases) and *c-KIT* (Curtin et al., 2006; Davies et al., 2002; Padua et al., 1985; Smalley et al., 2009). Activity in the MAPK pathway drives growth through the upregulation of cyclin D1 expression and the suppression of the cyclin dependent kinase inhibitor p27^{KIP1} (Bhatt et al., 2005; Smalley, 2003). Although much of the available evidence supports a role for the MAPK pathway in the uncontrolled proliferation of many cancer types, its potential role in the regulation of cell survival is less well characterized.

The PI3Ks are a family of lipid kinases that play a key role in regulating growth and survival (Cantley, 2002; Samuels and Velculescu, 2004; Wong et al.). Structurally, PI3K forms a heterodimer consisting of a p85 regulatory and a p110 catalytic subunit (Cantley, 2002). It is recruited to the membrane following the activation of receptor tyrosine kinases and associated adaptor proteins that bind to the SH2 domain of the p85 subunit (Cantley, 2002). The p110 domain can also be recruited and activated following the activation of Ras. Following membrane recruitment and activation, PI3K then phosphorylates the phosphatidylinositol-4,5,bisphosphate ring (PIP2) at the 3' position, converting PIP2 to PIP3. Once generated, PIP3 recruits and activates the downstream serine-threonine kinases PDK1 and AKT (Cantley, 2002). The AKT family consists of three members, AKT1-3 (Brazil et al., 2002), which exhibit different expression patterns depending upon cell type. AKT has a critical role in cancer development through its ability to regulate apoptosis via the direct phosphorylation of BAD, as well as effects upon many other pathways, including the stimulation of ribosomal S-6-kinase, the inhibition of Forkhead signaling and the inhibition of glycogen synthase kinase-3 (Datta et al., 1997; Robertson, 2005).

One of the most critical regulators of AKT, is the phosphatase and tensin homologue (PTEN), which degrades the products of PI3K, therefore preventing AKT activation (Salmena et al., 2008). Many cancers have constitutive activity in the PI3K/AKT signaling pathway and this can result from loss/mutation of *PTEN* (which often occurs in prostate, brain, breast cancers and melanoma), activating *Ras* mutations, mutations in PI3K and

activation/mutation of receptor tyrosine kinases (such as EGFR, PDGFR and HER2) (Cully and Downward, 2008; Salmena et al., 2008; Samuels et al., 2005; Samuels et al., 2004; She et al., 2008).

Recent studies have suggested that both the MAPK and AKT pathways may be good therapeutic targets in cancer (Carracedo et al., 2008; Engelman et al., 2008; Hoefflich et al., 2009; Smalley et al., 2006; Sos et al., 2009). A number of pharmaceutical companies and academic institutions are now developing small molecule inhibitors for both the MAPK and PI3K/AKT pathways. As these compounds move from pre-clinical to clinical development, there is a need to accurately quantify the levels of constitutive MAPK and AKT signaling activity in both pre-clinical cancer models and in clinical specimens. Work in our lab is focused upon targeted therapy strategies for melanoma, a tumor that has been shown to rely upon both MAPK and PI3K/AKT signaling (Smalley, 2010). In this chapter, we describe methods to measure constitutive MAPK and AKT activity at both the single-cell level (microscopy and flow cytometry) as well as in populations of cells (Western blotting). As melanoma cell lines typically have high levels of constitutive MAPK and PI3K/AKT signaling, we have used this system to examine the cell signaling effects of the BRAF-specific kinase inhibitor PLX4720, which is currently being evaluated as a novel anti-melanoma therapy (Flaherty et al., 2009; Tsai et al., 2008).

2. MAINTAINING MELANOMA CELL LINES

2.1 Culturing conditions

Prior to working with the cell lines, it is recommended to expand and freeze down a large stock of cells at an early passage and to perform DNA fingerprinting analysis in order to authenticate genetic identity. To ensure stability of the cell lines between experiments, we recommend replacing the cultures every 2 months with freshly thawed, early passage cells. For the experiments described herein, cells were maintained in a 5% CO₂, 37°C humidified incubator in growth media consisting of RPMI 1640 supplemented with 300mg/L L-glutamine, 5% heat inactivated fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. WM164 and 1205Lu melanoma cell lines were a kind gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). Most of the cell lines described below are available from The Coriell Institute (Camden, NJ). Further details can be found at <http://ccr.coriell.org/sections/Collections/Wistar>

2.1 Plating cells

ERK activity can be affected by cell density, therefore, it is important to plate the cells so that they remain sub-confluent over the course of the experiment. At the same time, an adequate number of cells should be seeded so that there is enough material for a clear read-out of the assay. We recommend a cell density of 60–70% confluency on the day of experimentation.

3. WESTERN BLOTTING

Signal transduction cascades are regulated through co-ordinated phosphorylation and dephosphorylation events (see (Pratilas et al., 2009) for an overview of the

phosphorylation events within the MAPK pathway). Constitutive activity in the MAPK and AKT signaling pathways can be easily assessed through the use of phosphorylation site-specific antibodies (aka phospho-specific antibodies). When used in traditional Western blotting, these antibodies allow for the sensitive detection of protein phosphorylation levels. In this section, we describe our technique for assessing ERK and AKT activity by Western blotting. Here we used Invitrogen's XCell SureLock Electrophoresis Cell for the separation of proteins and the XCell II Blot Module for the transfer of protein onto PVDF membrane, however, other equivalent apparatus can be used in place of the Invitrogen system. In the example shown in Figure 1, *BRAF* V600E mutated 1205Lu melanoma cells are treated with increasing concentrations of the BRAF inhibitor PLX4720 for 1 and 24 hrs. These data show that although PLX4720 blocks constitutive pERK signaling activity, this leads in turn to a rebound increase in pAKT signaling (Figure 1).

3.1 Harvesting cells and protein extraction

3.1.1 Materials

- RPMI growth media: RPMI 1640 supplemented with 300 mg/L L-glutamine, 5% heat inactivated fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin
 - 0.25% Trypsin-EDTA
 - 1 X PBS, (–) calcium chloride (–) magnesium chloride, pH7.4
 - Roche Complete, Mini, EDTA-free protease inhibitors cocktail (# 11 836 170 001).
 - RIPA lysis buffer: 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 24.1mM Na-deoxycholate, 0.1% SDS, 154mM NaCl, 1mM EDTA. Store RIPA buffer without protease inhibitors at 4°C. To make the modified RIPA buffer, take a 10 ml aliquot of RIPA lysis buffer and add 1 tablet of Roche Complete, Mini, EDTA-free protease inhibitors
 - 6 well tissue culture plates
 - 15 mL conical Falcon tubes
 - 1.5 mL Eppendorf tubes
1. Six well tissue culture plates were seeded with 1×10^5 cells per 2mL of growth media. Cells were grown overnight prior to treating with inhibitor.
 2. Prior to protein extraction, melanoma cell lines were treated for 1hr with DMSO or 0.03 to 3 µM of the BRAF inhibitor, PLX4720 (Plexxikon Inc, Berkley, CA). PLX4720 was dissolved in DMSO to make a 10 mM stock.
 3. Prior to harvesting the adherent melanoma cells, growth media containing non-adherent cells was first collected and placed in a Falcon tube. The cells were then rinsed with 1 mL of 1 X PBS per well. The 1 X PBS from the rinse was placed into the same Falcon tube containing the non-adherent cells. To detach the cells, 0.5 mL of 0.25% Trypsin-EDTA was added to each well. After 5 minutes of incubation at room temperature (RT), plates were gently rocked back and forth to detach the cells

followed by the addition of 1 mL of growth media to each well. Trypsinized cells were pipetted into the Falcon tube containing the non-adherent cells.

4. Falcon tubes containing the cells were centrifuged at 1500 rpm, 5 min, 4°C. The supernatant was aspirated off and discarded and the pellet was resuspended in 1 mL, cold 1 X PBS and transferred to a 1.5 mL Eppendorf tube.
5. Again, the cells were centrifuged at 1500rpm, 5 min, 4°C and the supernatant was aspirated off and discarded. Cells pellets were resuspended in 35 µL modified RIPA buffer and pipetted up and down several times followed by vortexing to disrupt the pellet and lyse the cells.
6. The cells were centrifuged at maximum speed (13,200 rpm), 30 min, 4°C. The soluble protein contained in the supernatant was collected and placed into new pre-chilled 1.5 mL Eppendorf tubes. Following protein extraction, cell lysates were stored at -20°C.

3.2 Determine Protein Concentration

3.2.1 Materials

- Pierce BCA Protein Assay kit and Instructions (Thermo Scientific Cat. #23225)
 - 96 well flat bottom Microplates
 - 37 °C incubator
 - 562 nm absorbance plate reader
1. Prepare the BSA standards according to the Standard Test Tube and Microplate Procedure using dH₂O as the diluent.
 2. Dilute the protein extracts 1:10 in dH₂O in a total volume of 25 µL per replicate and add 25 µL of standard or protein extract per well of a 96 well flat bottom Microplate.
 3. Prepare the BCA Working Reagent according to the instructions and add 200 µL per well containing standard or sample and incubate at 37°C for 30 min. Following incubation, allow the plate to cool down to RT and measure the absorbance at 562 nm.

3.3 Sample preparation

3.3.1 Materials

- 1.5 mL Eppendorf tubes
 - 2-Mercaptoethanol
 - NuPage (4X) LDS sample buffer (Invitrogen #NP0007)
 - Heat block set to 95 °C or boiling waterbath
- 1 To detect phosphorylation of ERK and AKT, we run our proteins under denatured, reduced conditions. Denaturing unfolds the protein to reveal the epitope of interest and confers an overall negative charge to the sample.

Therefore protein separation is based on molecular weight not on conformation of the protein.

- 2 The samples are generally run in 1 mm x 10 or 12 well gels and are prepared in a total volume of 10 to 15 μL . However, the maximum loading volume for a 12 well gel is 30 μL . In our constitutively active cell lines, 5 to 30 μg of total protein is sufficient for the detection of ERK phosphorylation. Detection of phospho-AKT usually requires 20 to 50 μg of total protein.
- 3 Samples are prepared in 1.5ml Eppendorf tubes following the recipe below:

20 μg protein (10 $\mu\text{g}/\mu\text{L}$ concentration)	2.0 μL
4x NuPage (4X) LDS sample buffer	2.5 μL
2-Mercaptoethanol (reducing agent)	0.1 μL
dH ₂ O	5.4 μL
Total Volume	10.0 μL

- 7 Heat the samples for 5 min at 95°C. Allow the samples to cool on at RT for 5–10 min. Centrifuge the samples briefly to remove condensation from the tops of the tubes. Load the samples within at least an hour after adding the reducing agent.

3.4 Electrophoresis

3.4.1 Materials

- 10 well Novex 8–16% Tris-Glycine Gels (Invitrogen, #EC6045BOX)
 - 12 well Novex 8–16% Tris-Glycine Gels (Invitrogen, #EC60452BOX)
 - 10 X Tris/Glycine/SDS buffer (Bio-Rad, #161-0732)
 - XCell *SureLock*® Mini-Cell Kit (Invitrogen, #EI0001) *Please refer to Invitrogen's manual for detailed instructions
 - Precision Plus Protein Kaleidoscope Standard (Bio-Rad, #161-0375)
1. Wipe off the pre-cast gel after removing it from the package and peel off the tape from the bottom of the cassette. You can also mark the wells with a sharpie marker to make them easier to see when you are loading the gel.
 2. Place the gel in the chamber with the notched side facing the middle compartment. If only 1 gel is being run, place a gel dam on the other side of the electrode. Snap the tension wedge to secure the gels in place and fill the middle chamber of the gel box with 500 mL 1 X Tris/glycine running buffer allowing the buffer to flow to the outer compartment.
 3. Remove the combs from the gels and load each sample with gel loading tips. Alongside the samples, load 10 μL of Bio-Rad's Kaleidoscope standard or another standard with a molecular weight range within the range of phospho-ERK and

phospho-AKT. Note that the molecular weight for phospho-ERK(1/2) is 42 and 44 kDa while the molecular weight for phospho-AKT is 60 kDa.

4. Separate the proteins by electrophoresing at 125V until the dye front reaches the bottom of the gel, this will usually take about 1.5 hrs.

3.5 Protein transfer

3.5.1 Materials

- Transfer buffer: 25 mM Tris Base, 192 mM Glycine, 20% methanol *Store the transfer buffer at 4°C
 - Polyvinylidene difluoride (PVDF) membranes
 - Shaker
 - Whatman filter paper
 - Pipet or 15mL conical tube
 - XCell II™ Blot Module CE Mark (Invitrogen, #EI9051) *Please refer to Invitrogen's manual for further detailed instructions
 - Sponge pads for blotting (Invitrogen, #EI9052)
 - Power supply
1. Pre-wet the PVDF membrane for 30 sec in methanol. Pour off the methanol and add 10–20 mL transfer buffer and shake for 5 mins. Soak the filter paper briefly in transfer buffer immediately prior to use.
 2. Open the gel cassette, the notched side of the cassette should face up. Carefully remove and discard the top plate, gel remains in the bottom slotted plate. Remove wells with the gel knife.
 3. Place a piece of transfer buffer soaked filter paper on top of the gel and lay just above the slot in the bottom of the cassette, leaving the “foot” of the gel uncovered. Keep the filter paper saturated with the transfer buffer and remove all trapped air bubbles by gently rolling over the surface using a pipette or 15ml conical tube as a roller.
 4. Turn the plate over so the gel and filter paper are facing downwards over a flat surface. Use the gel knife to push the “foot” out of the slot allowing the gel to be released from the plate. Place the gel on a flat surface and cut off the “foot” of the gel with a gel knife.
 5. Wet the surface of the gel with transfer buffer and place the pre-soaked transfer membrane on top of the gel. Use a pipette or conical tube to roll out all air bubbles. Place another pre-soaked filter paper on top of the membrane and again ensure that all trapped air bubbles are removed.
 6. Place two transfer buffer soaked blotting pads into the deeper cathode (–) core of the blot module. Place the gel membrane assembly on top of the blotting pads in the same sequence making sure that the gel is closest to the cathode core.

7. Add more pre-soaked blotting pads to the assembly so that the pads rise approximately 0.5 cm over the rim of cathode core. Place the anode (+) core on top of the pads.
8. Place the gel/membrane assembly core into the chamber and lock the core into place with the Gel Tension Wedge.
9. Fill the blot module with transfer buffer until the gel/membrane assembly is just covered in buffer. Do not fill all the way to the top as this will only generate extra conductivity and heat.
10. Fill the outer buffer chamber with deionized water until it reaches approximately 2 cm from the top of the lower buffer chamber and place the lid on top of the unit. Transfer at 4°C (cold room) at 35V for 1hr or 12V overnight.

3.6 Antibody incubations and Western blot development

3.1 Materials

- 10 X TBST (recipe for 1L, pH7.6) Add 12.11g Tris Base and 87.66g NaCl to 800mL ddH₂O. Stir to completely dissolve the Tris and NaCl. Adjust the pH to 7.6 with 10N HCl. Add 10 ml Tween 20 and stir to dissolve. Adjust the final volume to 1 L with ddH₂O.
 - 1 X TBST: Dilute 10xTBST to 1X with ddH₂O
 - Blocking buffer: 5% non-fat milk in 1 X TBST
 - Primary antibody dilution buffer: 5% BSA in 1 X TBST
 - Phospho-p44/42 MAPK (ERK1/2) antibody (Cell Signaling Technologies, #4370)
 - p44/42 MAPK (ERK1/2) antibody (Cell Signaling Technologies, #4695)
 - Phospho-AKT (Ser473) antibody (Cell Signaling Technologies, #4058)
 - Phospho-AKT (Thr308) antibody (Cell Signaling Technologies, #4056)
 - AKT antibody (Cell Signaling Technologies, #9272)
 - Goat anti-rabbit IgG-HRP (GE Healthcare, #RPN4301)
 - ECL Western Lightning (Perkin Elmer, #NEL100001EA)
 - HyBlot CL Autoradiography Film (Denville Scientific Inc, #E3012)
 - Autoradiography Cassette (Fisher Scientific, #FBCS 57)
 - X-ray film developer
 - Restore Western Blot Stripping Buffer (Thermo Scientific, #21059)
1. Rinse the PVDF membrane briefly with methanol and allow to air dry for ~15 min on top of a clean piece of filter paper.
 2. Once dry, place the membrane into blocking buffer and shake at RT for 1hr.
 3. Briefly rinse the membrane in 1 X TBST.

4. Make 1:1000 and 1:500 dilutions of the primary rabbit anti-phospho-ERK and phospho-AKT antibodies, respectively.
5. Incubate in primary ab solution overnight at 4°C (while shaking).
6. Wash 3 times 10 min, RT on the shaker with 1 X TBST
7. Prepare the secondary antibody (anti-rabbit IgG-HRP) in blocking buffer at a 1:2000 dilution.
8. Incubate in secondary ab, 1hr Room Temperature. Wash 3 times 10 min, RT on the shaker w/1 X TBST
9. Remove excess wash buffer by touching one corner of the membrane over a paper towel.
10. Place the membrane onto a plastic cover or saran wrap.
11. Wet the membrane with 1ml of a 1:1 mix of Western Lightning ECL Reagents. Incubate at room temperature for 1 min.
12. Cover the membrane with a plastic cover or saran wrap and wipe off the excess ECL reagent.
13. Tape the encased membrane onto the inside of a cassette film holder.
14. Expose the film by placing it on top of the membrane and develop it in an x-ray developer. In order to increase the chances of detecting the protein of interest while avoiding overexposure of the film, it is best to carry out a range of exposures times.
15. To ensure even protein loading, the membrane should be stripped and reprobed for the total protein corresponding to the phosphorylated protein.
16. Briefly rinse the blot with 1X TBST and cover the blot with Restore Western Stripping Buffer. Shake at RT for 15 min.
17. Repeat the wash with 1X TBST and place the blot into a new container. Immerse the blot in blocking buffer and incubate at RT for 1hr. Briefly rinse in 1 X TBST.
18. For total ERK and total AKT, make 1:1000 dilutions in 5% BSA 1 X TBST Repeat steps 5 to 14.

4. PHOSPHO-FLOW CYTOMETRY

Phospho-flow cytometry allows for quantification of phosphorylation at the single cell level. In addition, the flow cytometry platform through the combination of multiple antibodies, allows for visualization of subpopulations of cells as well as multiple signaling events with a single cell or population. A sample experiment showing the ability of the BRAF inhibitor PLX4720 to inhibit constitutive pERK activity in two *BRAF* V600E mutated melanoma cell lines is shown in figure 2.

4.1 Materials

- anti-pERK1/2 (T202/Y204)-AF647 (BD Biosciences, #612593)

- mouse IgG1,k-AF647 (BD Biosciences, #557732)
 - BD Phosflow Fix Buffer I (BD Biosciences, #557870)
 - BD Phosflow Perm/Wash Buffer I (BD Biosciences, #557885)
 - Staining buffer: 0.2% BSA, 1xPBS, pH 7.4
 - FACS tubes (BD Falcon, #352235)
 - BD FACSCalibur or other similar instrument equipped with a 635 or 638nm laser capable of exciting AF647.
1. Melanoma cell lines were seeded at 2×10^6 cells per well in 6-well tissue culture plates and allowed to attach overnight.
 2. The following day, growth media (5% FBS, RPMI+L-glutamine) was replaced and individual wells containing cells at ~50% confluency were treated with 0.03, 0.3, 3 or 30 μ M PLX4720. For vehicle controls, DMSO was added at an equivalent volume to the 30 μ M PLX treated wells.
 3. The cells were treated for 1 hr at which time non-adherent and adherent cells were collected as described in the Western blotting section.
 4. Following collection, cells were immediately fixed with an equivalent volume of Phosflow Fix Buffer I for 10 min at 37°C.
 5. Fixed cells were pelleted and resuspended in Phosflow Perm/Wash Buffer I and permeabilized for 30 min on ice.
 6. Cells were washed with staining buffer (0.2% BSA, 1X PBS, pH 7.4) and counted.
 7. Cells from each concentration of treatment were individually stained with pERK or the isotype control antibody. In addition, cells from each treatment were pooled and left unstained in order to set up the scatter and negative parameters of the flow cytometer.
 8. A total of 1×10^5 cells in 100 μ L staining buffer was stained with 3 μ L pERK1/2-AF647 antibody or 1.25 μ L pERK1/2 isotype control, mouse IgG1,k-AF647. Cells were incubated in the dark for 1hr at RT.
 9. Following staining, cells were washed with 1 mL staining buffer and resuspended in 250 μ L staining buffer.
 10. At least 30,000 events were acquired on a FACSCalibur flow cytometer.
 11. Analysis was carried out with FlowJo v8.7.1 software (Tree Star, Stanford, CA). A separate gate was generated for each treatment concentration by gating on approximately the 99th percentile of the corresponding isotype control. In order to delineate positive stained cells, the isotype control gate, which represents the negative staining gate, was then copied to the pERK stained sample.

5. Immunofluorescence

An alternate method for measuring constitutive MAPK and AKT activity in individual melanoma cells is to stain cell cultures with phospho-specific antibodies against either ERK or AKT and then use immunofluorescence microscopy to visualize and count the number of cells with pathway activity. Although the method requires the manual inspection of cultures, it allows rare events to be captured that may be missed by flow cytometry and is also well suited for examining nuclear vs cytoplasmic localization of the signals. There is evidence from immunohistochemical staining of melanoma samples that the intensity of pERK staining is heterogeneous within tumors and that levels of pERK expression can vary between the different cellular compartments (example shown in Figure 3). In the method outlined below, melanoma cells are seeded sub-fluently onto glass coverslips and incubated overnight in the absence or presence of the BRAF inhibitor PLX4720. Cells are then fixed in paraformaldehyde, permeabilized and stained for phospho-ERK. In the example shown in Figure 4, increasing concentrations of the BRAF inhibitor PLX4720 reduces the phospho-ERK staining of the melanoma cell cultures.

3.1 reagents

4% paraformaldehyde solution

- dilute 10 ml of 16% paraformaldehyde solution (Electron Microscopy Systems, Hatfield, PA.) in 30 ml of PBS in a 50 ml Falcon Tube. Cover the tube with foil and store in the dark. The diluted paraformaldehyde can be used for up to 14 days.

0.2 % v/v Triton-X-100 solution

- Make a 10% v/v Triton solution in PBS by adding 2 ml of stock Triton X-100 to a 50 ml Falcon Tube. Make this up to a total final volume of 20 ml PBS. Vortex to ensure the Triton and PBS are thoroughly mixed. The resulting solution should be clear with no traces of un-dissolved Triton. Make a working stock of 0.2 % v/v Triton by adding 0.2 ml of 10% v/v Triton solution to a Falcon tube and making to a final volume of 10 ml in PBS.

1% BSA-PBS blocking solution—Dissolve 0.2 g of Bovine Serum Albumin (BSA, Fraction V) in 20 ml of PBS.

Primary antibodies—For dual pERK/pAKT staining, we use mouse anti-phospho-ERK (Cell Signaling technology #9106) and either Rabbit Ser473 AKT (Cell Signaling Technology, #4058) or anti-Thr308 AKT (Cell Signaling Technology, #2965).

Secondary antibodies—Anti-mouse Alexa Fluor 488 (Invitrogen, #A21141)

Anti-Rabbit Alexa Fluor 594 (Invitrogen, #A11012).

5.2 Preparing the cells on coverslips

1. Place 1 glass coverslip into 1 well of a 6-well plate and immerse in 70% ethanol. Remove the ethanol using a vacuum line and allow the coverslips to air dry. Note:

It is important for all of the ethanol to evaporate and the coverslips to be completely dry before proceeding to the next stage.

2. Remove the cells from the flask by removing the media and adding 3 ml of Trypsin for 5 minutes. Once the cells have detached, add 7 ml of media and centrifuge at 1500 rpm for 5 minutes.
3. Remove the media and re-suspend the cells in 10 ml of fresh media. Perform cell counts using a hemocytometer and re-suspend the cells at a dilution of 50,000 cell/ml.
4. Add 100 μ L of cell suspension to the center of each coverslip and return the plates to the cell culture incubator and incubate at 37°C for 15 minutes or until the cells have adhered.
5. Add 2 ml of media to each well of the plate and leave the cultures to equilibrate overnight.
6. Add either vehicle (DMSO), or increasing concentrations of the pharmacological inhibitor of choice (in Figure 4 the BRAF inhibitor PLX4720 is used) for 1–24 hrs.

5.3 Fixing and staining the cells

1. Remove media using a vacuum line and add 1 ml of 4% paraformaldehyde per well (15 minutes). Once fixed, the coverslips can either be kept in the 6-well plates (wrapped in parafilm) and stored 4°C or used immediately.
2. Remove the formaldehyde and wash the cells twice with PBS.
3. Remove PBS and add 1 ml 0.2% v/v of Triton X-100 for 5 minutes to permeabilize the cells.
4. Remove Triton and wash the coverslips 2X with PBS.
5. Add 1 ml of PBS containing 1% BSA and leave to block for 15 mins at RT.
6. Prepare the staining surface by laying the inverted lid of the 6-well plate flat on the bench and placing parafilm on top of this.
7. Make up primary antibody at a concentration of 1:20–1:40 in BSA/PBS. Allow 50 μ L of BSA-PBS per coverslip. Place 50 μ L on top of the parafilm, before carefully removing a coverslip using a pair of fine forceps. Gently blot the liquid off the coverslip before inverting the coverslip (so that the cells are facing downwards onto the antibody) onto the 50 μ L of BSA/PBS antibody solution. When you have performed this process for each of the coverslips, cover the lid with foil and place in a cell culture incubator (37°C) for 1 hour.
8. Return the coverslips to 6-well plate and wash 2X in PBS over 30 minutes at room temperature.
9. Repeat steps 6–8 for the secondary antibody (1:200) in BSA/PBS and again incubate at 37°C for 1 hour.

10. Wash the coverslips 2X in PBS and once in distilled water (to remove salt deposits) and blot the coverslip gently on a Kim Wipe (or other tissue paper).
11. Put a single drop of anti-fade (we use Vectashield with DAPI) onto a glass slide and invert the coverslip gently on top of it (so the cells are in contact with the Vectashield). Seal the edges of the coverslip using nail polish.
12. Image the slides using an upright fluorescence microscope.

6. Conclusions

Measuring constitutive levels of MAPK and PI3K/AKT signaling is an essential part of assessing the activity of novel, targeted anti-cancer therapies. In this chapter we have outlined methods for looking at MAPK/AKT activity in mass cell cultures using Western blotting, as well as techniques to quantify signaling events across cell populations (flow cytometry and immunofluorescence). In the examples given in Figures 1–4 these methods were used to investigate the ability of the novel BRAF-specific kinase inhibitor PLX4720 to inhibit constitutive MAPK signaling in *BRAF* V600E-mutated human melanoma cell lines (Tsai et al., 2008). Although Western blotting is regarded as being the Gold Standard for signal transduction studies it is limited in giving only a “snap-shot” of the sum total of signaling activity within the whole cell population. There is a growing realization that Western blotting methods are not adequate at capturing the cell signaling heterogeneity that is commonly observed within populations of adherent cancer cells. Quantification of signaling at the individual cell level is critical to understand drug resistance and therapy escape, with recent studies suggesting that even minor differences in signaling dynamics within clonal populations of cancer cells lead to vastly different therapeutic outcomes (Cohen et al., 2008; Gascoigne and Taylor, 2008). Flow cytometry methods that utilize phospho-specific antibodies to measure signaling at the single cell level, are rapidly gaining popularity amongst those working upon non-adherent cell populations, such as immune cells and leukemia (Kotecha et al., 2008). In future, as the methods are refined we expect phospho-Flow techniques to be more widely adopted by those working on adherent tumor cell lines. We further expect that enhanced methods to quantify signaling heterogeneity will bring important new insights into our understanding of constitutive MAPK and AKT signaling within cancer populations and that this will in turn lead to the development of optimized small molecule inhibitors for targeting signal transduction pathways in cancer.

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Figure 1.

Measuring constitutive pERK and pAKT activity by Western blotting. 1205Lu melanoma cells were treated with either vehicle (DMSO, 0) or increasing concentrations of PLX4720 (0 – 3 μM, 1hr for pERK and 24 hr for pAKT). Protein was then extracted, resolved by Western blotting and probed for expression of pAKT (Ser473 and Thr308) and pERK. Equal protein loading was demonstrated by stripping the original blot and reprobing for either total AKT or total ERK.

Figure 2.

Using phospho-flow to measure constitutive pERK activity. A). Demonstration of the specificity of response. WM164 melanoma cells were treated with PLX4720 (0 – 3 μ M, 1 hr), fixed and stained for either pERK or a matching Rabbit IgG isotype control. Increasing concentrations of drug were found to inhibit pERK staining. The isotype control was used to gate for the analysis shown in Figure 1B. B). Quantification of levels of pERK signaling following PLX4720 treatment as measured by phospho-flow cytometry. Cell lines (WM164 and 1205Lu melanoma cell lines) were treated with PLX4720 (0 – 3 μ M, 1 hr), fixed and stained for pERK. MFI (Median Fluorescence intensity) indicates the absolute level of pERK staining.

Metastatic melanoma

Figure 3.

pERK is located in both the nuclear and cytoplasmic compartments of human melanoma metastases. Figure shows a representative clinical specimen of human melanoma stained for pERK by immunohistochemistry. Arrows indicate nuclear expression of pERK. Magnification: X200.

Figure 4.

PLX4720 reduced the expression of pERK. WM164 cells were treated with increasing concentrations of PLX4720 (0.03 – 30 μ M, 1 hr) before being fixed, permeabilized and stained for pERK. Scale bar = 20 μ M.