2017

Quantification of Cell Signaling Networks Using Kinase Activity Chemosensors

Jon R. Beck
University of Nebraska - Lincoln

Edward N. Harris
University of Nebraska - Lincoln, eharris5@unl.edu

Cliff I. Stains
University of Nebraska-Lincoln, cstains2@unl.edu

Quantification of Cell Signaling Networks Using Kinase Activity Chemosensors

Jon R. Beck, Edward N. Harris, and Cliff I. Stains

University of Nebraska–Lincoln

Abstract

The ability to directly determine endogenous kinase activity in tissue homogenates provides valuable insights into signaling aberrations that underlie disease phenotypes. When activity data is collected across a panel of kinases, a unique "signaling fingerprint" is generated that allows for discrimination between diseased and normal tissue. Here we describe the use of peptide-based kinase activity sensors to fingerprint the signaling changes associated with disease states. This approach leverages the phosphorylation-sensitive sulfonamido-oxine (Sox) fluorophore to provide a direct readout of kinase enzymatic activity in unfractionated tissue homogenates from animal models or clinical samples. To demonstrate the application of this technology, we focus on a rat model of nonalcoholic fatty liver disease (NAFLD). Sox-based activity probes allow for the rapid and straightforward analysis of changes in kinase enzymatic activity associated with disease states, providing leads for further investigation using traditional biochemical approaches.

Keywords: Kinase activity assay, Cell signaling, Nonalcoholic fatty liver disease, Phosphorylation, Fluorescence-based biosensor

1 Introduction

Quantification of kinase enzymatic activity has traditionally been accomplished by monitoring the transfer of radioactive phosphate from [γ-^{32}P]ATP to a peptide or protein substrate [1–3]. While powerful in terms of general applicability across the kinome, this method of activity determination may be difficult to implement in samples containing multiple kinases, such as tissue homogenates, due to the

inherent promiscuity of [γ-^{32}P]ATP. Additionally, the use of radioactivity presents potential hazards to users and requires specialized training as well as dedicated equipment. In contrast, peptide-based substrates that rely on a change in fluorescence upon phosphorylation as a readout of kinase activity present an alternative for the straightforward analysis of kinase activity [4]. Such probes may be used to directly report on kinase enzymatic activity in unfractionated cell lysates or tissue homogenates, provided that sufficiently selective peptide substrates can be identified [5–7]. Here, we describe the use of peptide substrates that utilize the phosphorylation-sensitive Sox fluorophore to report on kinase activity. Sox-based probes produce an increase in fluorescence (ex = 360 nm, em = 485 nm) upon phosphorylation, through a process termed chelation-enhanced fluorescence (CHEF) [8]. Controlling the concentration of Mg^{2+} in the assay allows for discrimination between the phosphorylated and nonphosphorylated probe since binding affinities for Mg^{2+} generally improve by an order of magnitude upon phosphorylation. First-generation Sox sensors relied on a β-turn motif to provide efficient Mg^{2+} binding upon phosphorylation [9, 10]. Though powerful, incorporation of the β-turn motif resulted in removal of the substrate sequence either N- or C-terminal to the site of phosphorylation. In order to improve kinase selectivity, a second-generation approach was described in which a single amino acid in a peptide substrate is mutated to cysteine. Subsequent alkylation with the Sox fluorophore results in generation of a CSox-based probe with a single amino acid mutation relative to the wild-type sequence (**Fig. 1a**) [11]. Due to the increased flexibility of the cysteine side chain, efficient Mg^{2+} binding upon phosphorylation is retained in CSox-based sensor constructs. Moreover, kinetic parameters for target kinases can be significantly improved [11]. Recent work has identified a panel of CSox-based kinase activity probes for p38α, MK2, Akt, ERK1/ERK2, and PKA (**Table 1**) [11–13] that can be utilized to interrogate signaling changes in unfractionated cell lysates or tissue homogenates (Fig. 1b) [5]. In some cases, selectivity is achieved by utilizing broad spectrum inhibitors to suppress the activity of off-target enzymes while preserving signal from the target enzyme [5, 13, 14]. In addition to these validated probes (Table 1), numerous Sox-based substrates capable of reporting on kinase activity in vitro have been reported, including sensors for focal adhesion kinase [15], leucine-rich repeat kinase 2
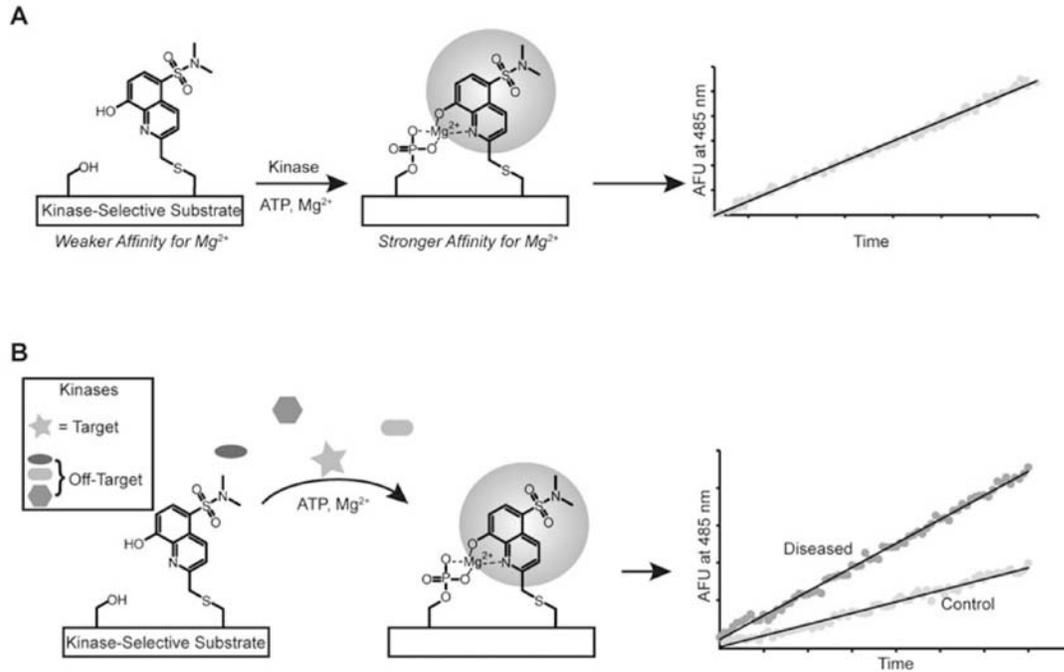
Fig. 1. Monitoring endogenous kinase activity with CSox-based chemosensors. **(a)** The Sox fluorophore is incorporated near the site of phosphorylation on a known peptide substrate that is selective for the kinase of interest. Following phosphorylation by the kinase of interest, the Sox fluorophore undergoes CHEF in the presence of Mg^{2+}. The increase in signal due to CHEF is measured over time. The resulting data is proportional to the rate of phosphorylation of the probe. **(b)** Selective CSox-based chemosensors can resolve the activity of a target kinase in the presence of off-target enzymes. Differences in kinase activity can be readily quantified between disease and normal phenotypes

Table 1. CSox-based chemosensors used in this protocol

Kinase	Substrate sequence	Pathway	Reference
p38α	RKPDLRVVIPP-(AOO)3-QP-CSox-ASPVV[a]	Inflammation	[13]
MK2	AHLQRQLSI-CSox-HH	Inflammation	[11]
PKA	ALRRASL-CSox-AA	Metabolism	[11]
Akt	ARKRERAYSF-CSox-HHA	Survival	[11]
ERK1/ERK2	VP-CSox-LTPGGRRG-PNT Domain(S46-K138)[b]	Growth	[12]

a. AOO is an 8-amino-3,6-dioxaoctanoic acid linker
b. This probe is synthesized by ligating a CSox-containing peptide to a protein domain termed PNT

[16], rho-associated protein kinase [17], and spleen tyrosine kinase [18, 19]. Further optimization of these probes for use in unfractionated cell lysates or tissue homogenates will allow for broader coverage of signaling pathways. Beyond detection of protein kinase activity, we have recently demonstrated that CSox-based probes can be repurposed to detect the activity of endogenous protein phosphatases [20]. This battery of validated CSox-based assays allows for the fundamental investigation of signaling perturbations associated with disease states.

In this chapter, we provide detailed protocols for generating tissue homogenates that preserve endogenous kinase activity as well as instructions for assay setup and data analysis. As a case study, we include data generated from a rat model of NAFLD [21, 22] to illustrate the application of this methodology. In addition to rat liver tissues, this protocol has been used to analyze kinase activity in several other tissue types including the human breast, lung, and prostate [5]. Lastly, this general procedure may be adapted for new CSox-based probes [23] as they become available.

2 Materials

2.1 Tissue Homogenate Preparation Components

1. Ice.
2. 2.0 ml screw cap cryotubes.
3. 15 ml conical tubes.
4. 1000 ml micropipette with tips.
5. Cold PBS.
6. Lysis buffer: 50 mM Tris–HCl, pH 7.5 at 22 °C, 150 mM NaCl, 100 μM Na_3VO_4, 1% Triton X-100, 1× Protease Inhibitor Cocktail III (EMD Millipore Cocktail, 539134), 1× Phosphatase Inhibitor Cocktail III (Sigma, P0044), 1 mM DTT, and 2 mM EGTA. Store up to 6 months at –20 °C.
7. Homogenizer.
8. Temperature-controlled microcentrifuge.
9. 1.7 ml microcentrifuge tubes.

10. Liquid nitrogen.

11. Low-form Dewar flask.

12. 0.7 ml microcentrifuge tubes.

2.2 Kinase Activity Assay Components

1. Total protein assay.

2. Fluorescence microplate reader.

3. 384-well, half-area, white microplate (Corning, 3824).

4. 10× Master Mix: 500 mM Tris–HCl, pH = 7.5 at 22 °C, 20 mM EGTA, 0.1% Brij-35, 10 mM DTT, 100 mM $MgCl_2$, and 10 mM ATP (store at –20 °C).

5. 10× kinase activity sensor (all dissolved in nanopure H_2O) (*see* Note 1):

      (a) 25 µM Akt chemosensor.

      (b) 10 µM p38α chemosensor.

      (c) 25 µM MK2 chemosensor.

      (d) 100 µM PKA chemosensor.

      (e) 50 µM ERK1/ERK2 chemosensor.

6. 10× inhibitor cocktails (diluted in DMSO; store at –20 °C) (*see* Note 2):

      (a) Akt: 40 µ MPKC inhibitor peptide, 40 µM calmidazolium, and 50 µM GF109203X.

      (b) p38α: 10 µM staurosporine (and 10 µM SB203580 in p38α-inhibited wells; *see* Note 3).

      (c) MK2: no inhibitors required.

      (d) PKA: 40 µM PKC inhibitor peptide, 40 µM calmidazolium, and 50 µM GF109203X.

      (e) ERK1/ERK2: no inhibitors required.

7. Multichannel pipette.

8. Ice.

9. Lysis buffer: *see* component 6, Subheading 2.1.

3 Methods

3.1 Preparation of Tissue Homogenates

1. Following tissue extraction, samples should immediately be placed in cold screw cap cryotubes, labeled, and flash frozen in liquid nitrogen.

2. Flash-frozen samples should then be stored at –80 °C until they are homogenized (*see* Note 4).

3. Prior to removing cryotube samples from the –80 °C freezer, weigh 15 ml conical tubes sufficient for the number of samples that are to be homogenized, and place the conical tubes on ice.

4. Remove the frozen cryotube samples one at a time, and, using a clean razor blade, cut off approximately 100 mg of tissue and place in a chilled conical tube. Place the conical tube back on ice, and return any unused sample to its cryotube and immediately return to the –80 °C freezer.

5. Repeat step 4 until all of the samples have been placed in conical tubes.

6. Weigh each conical tube again and determine the mass of each sample.

7. To each sample, add 2 ml of cold phosphate-buffered saline (PBS) to wash the samples (*see* Note 5).

8. Remove and discard the used PBS with at 1000 μl pipette.

9. Repeat steps 7 and 8 until the PBS wash is clear.

10. Add lysis buffer to each tissue sample (3 μl/mg).

11. Homogenize each sample in lysis buffer on ice (*see* Note 6).

12. Allow the samples to incubate for 15 min on ice.

13. While the samples are incubating, cool a microcentrifuge to 4 °C, and label a sufficient number of 1.7 ml microcentrifuge tubes for each sample. Place the empty tubes on ice.

14. Using a 1000 μl micropipette, transfer each homogenized sample from their respective conical tubes to the appropriately labeled microcentrifuge tubes, and return to the ice.

15. After all of the samples have been transferred to microcentrifuge tubes, place the tubes in the cooled microcentrifuge, and centrifuge for 5 min at 15,000 rcf.

16. While the samples are centrifuging, label additional 1.7 ml microcentrifuge tubes for each sample, and place on ice.

17. Remove the homogenates from the microcentrifuge and return them to the ice.

18. Using a 1000 μl pipette, carefully remove the soluble fraction from each tube, and place in the appropriately labeled new microcentrifuge tube from step 16. Discard the pellets (*see* Note 7). For storage of the samples, follow steps 19–21; otherwise, proceed to Subheading 3.2.

19. Add liquid nitrogen to a low-form Dewar flask.

20. For each sample, aliquot 10 μl of the soluble fraction to labeled 0.7 ml microcentrifuge tubes until the sample has all been aliquoted. Immediately flash freeze the aliquoted samples in the liquid nitrogen.

21. Following flash freezing, place all samples in a –80 °C freezer for storage.

3.2 Quantifying Kinase Activity
in Tissue Homogenates

1. Determine the total protein concentration of each homogenized sample using a total protein assay (*see* Note 8).

2. Turn on the microplate reader and equilibrate to 30 °C.

3. Obtain a 384-well microplate (*see* Subheading 2.2).

4. Add 4 μl of 10× Master Mix into each well intended to be used for activity determination. The assay should be conducted in triplicate for each sample. Due to the off-target subtraction required for p38α analysis, six wells will be needed per sample for this kinase: three for p38α activity determination and three for background subtraction.

5. Add 4 μl of the desired 10× Kinase Activity Sensor to each well.

6. Add 4 μl of 10× inhibitor cocktail as required for the specific kinase activity assay.

7. Dilute each well to 36 μl total volume with nanopure H_2O.

8. Mix the wells with a multichannel pipette, displacing ~20% of the well volume per mix.

9. Place the plate into the microplate reader for 10 min to equilibrate the assay components to the assay temperature.

10. Following equilibration, collect fluorescence data on the wells for 10 min (1 min between reads, λ_{ex} = 360 nm, λ_{em} = 485 nm) in order to identify any aberrant wells prior to activity determination. Wells with significantly different fluorescence readings (10%) should be discarded, and another well should be prepared.

11. While the plate is equilibrating and being read, remove one aliquot for each sample to be assayed from the –80 °C freezer, and place on ice.

12. Dilute each sample with cold lysis buffer to the ideal concentration for the kinase being assayed (0.83 μg/μl for Akt, p38α, and MK2, 1.67 μg/μl for PKA, and 3.33 μg/μl for ERK1/ ERK2) (*see* Note 9).

13. Following the 10 min read period, remove the plate from the microplate reader, and add 4 μl of diluted homogenate per well based on the kinase being assayed.

14. Following the addition of homogenate to all wells, quickly mix the assays with a multichannel pipette by displacing ~20% of the total volume multiple times (*see* Note 10).

15. Place the plate back in the microplate reader, and read for 2 h using the same conditions as in step 10 (*see* Note 11).

16. Following the completion of the assay, remove the plate and cover all unused wells to protect them from accumulation of particulate matter (*see* Note 12).

17. The initial linear portion of the kinetic data for each sample can now be fit to a linear line (**Fig. 2a**). The slope of this line is proportional to the reaction rate (*see* Note 13).
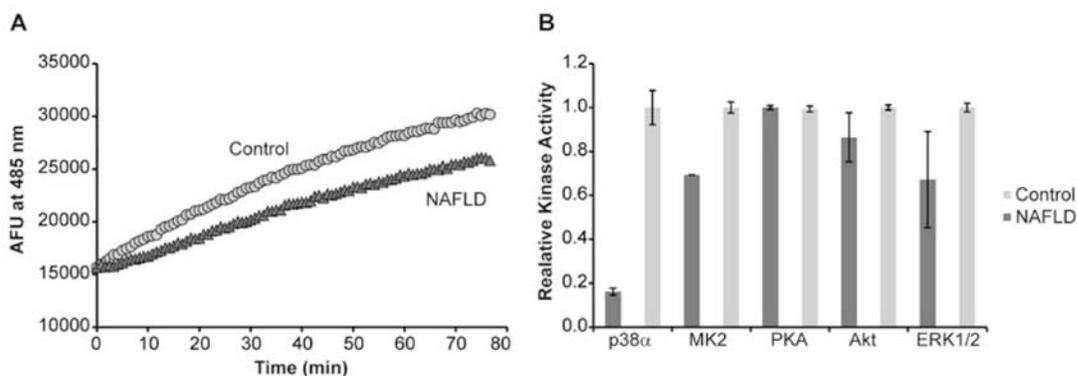
Fig. 2. Analysis of kinase activity in a rat model of NAFLD. **(a)** Representative raw data for an MK2 assay using rat liver homogenates. A clear decrease in the rate of phosphorylation of the MK2 probe is observed in NALFD (triangles) versus control (circles) homogenates. **(b)** A profile of kinase activity from NAFLD and control liver homogenates ($n = 6$ animals, assays run in triplicate for each animal). Activities are presented relative to respective control reactions for each enzyme

18. Average the reaction slopes of each triplicate sample set, and calculate the standard deviation. Significance can be determined using a two-tailed student's *t*-test. For the p38α assay, the average slope of the SB203580-containing wells should be background subtracted from the average slope of wells not containing SB203580 in order to obtain the activity due to p38α.

19. The average reaction slope can also be determined among groups of similar homogenates (diseased vs. control) by averaging the mean of each triplicate experiment across each sample type (Fig. 2b).

4 Notes

1. The synthesis of each validated kinase chemosensor has been described in the literature [11–13]. Alternatively, custom Sox-based peptides are currently available from AssayQuant Technologies Inc. Additional CSox-based chemosensors that have been validated for use in complex biological systems [23] could be used similarly to those described in this protocol.

2. The suggested concentrations are such as to provide for 10% DMSO (v/v) in the final assay. We have found that up to 10% (v/v) of DMSO does not significantly alter kinase activity.

3. The p38α assay requires two reactions to be run: (a) inhibited with staurosporine (allows for p38α detection and suppression of some but not all off-target activity) and (b) inhibited with both staurosporine and SB203580 (inhibits p38α activity but not remaining off-target activity). Background subtraction of wells with SB203580 from wells without allows for resolution of p38α activity [13].

4. These tissue samples can be stored for over 6 months without appreciable loss of kinase activity.

5. This step removes excess blood and fluid from the tissue sample.

6. Our laboratory typically uses a handheld tissue homogenizer (Omni, TH115-PCRH) with disposable, hard tissue tips (Omni, 30750H). Other types of homogenizers may be used. Homogenization times should be kept as short as possible in order to avoid excessive sample heating. Use short bursts from the homogenizer until the tissue has been sufficiently broken down.

7. After centrifugation, a thin, cloudy lipid layer may appear on top of the soluble fraction. Avoid collecting the lipid layer by piercing it with the pipette tip before beginning to collect the soluble fraction. Once the soluble fraction has been collected and transferred to the new 1.7 ml microcentrifuge tube, mix with the pipette to increase the homogeneity of the sample.

8. Once a sample has been thawed for any reason, it should not be refrozen and used for activity assays. Freeze-thawed samples can be saved and used for Western blotting.

9. These concentrations allow for the optimized final total protein to be added to each well: 3.3 μg for Akt, p38α and MK2; 6.7 μg for PKA; and 13.2 μg for ERK1/ERK2.

10. Take care not to introduce bubbles when adding diluted homogenate to the assay wells as well as when mixing. Avoid this by not injecting air into the mixture. Large bubbles may be dispersed with a hypodermic needle, but small bubbles are difficult to remove and will adversely influence the assay results.

11. Comparison of fluorescence to wells prior to addition of lysate allows for identification of reactions in which a large proportion of substrate turnover has occurred prior to reading. In order to obtain the linear region of the reaction, less lysate may be used.

12. Plates may be used multiple times, but individual assay wells should not be reused.

13. At times, there may be a lag phase before the fluorescence intensity begins to increase [14]. If this phenomenon occurs, only fit the linear trend line to the data after the increase is observed. Furthermore, if the data becomes hyperbolic as the reaction proceeds, only fit a linear trend line to the initial linear portion of the reaction curve. In all cases, the same time period should be used for each sample assaying the same kinase, so as to maintain consistency in the data.

References

1. Hastie CJ, McLauchlan HJ, Cohen P (2006) Assay of protein kinases using radio-labeled ATP: a protocol. Nat Protoc 1:968–971

2. Witt JJ, Roskoski R (1975) Rapid protein kinase assay using phosphocellulose-paper absorption. Anal Biochem 66:253–258

3. Glass DB, Masaracchia RA, Feramisco JR, Kemp BE (1978) Isolation of phosphorylated peptides and proteins on ion-exchange papers. Anal Biochem 87:566–575

4. Gonzalez-Vera JA (2012) Probing the kinome in real time with fluorescent peptides. Chem Soc Rev 41:1652–1664

5. Stains CI, Tedford NC, Walkup TC, Lukovic E, Goguen BN, Griffith LG, Lauffenburger DA, Imperiali B (2012) Interrogating signaling nodes involved in cellular transformations using kinase activity probes. Chem Biol 19:210–217

6. Wang Q, Zimmerman EI, Toutchkine A, Martin TD, Graves LM, Lawrence DS (2010) Multicolor monitoring of dysregulated protein kinases in chronic myelogenous leukemia. ACS Chem Biol 5:887–895

7. Warthaka M, Adelmann CH, Kaoud TS, Edupuganti R, Yan C, Johnson WH Jr, Ferguson S, Tavares CD, Pence LJ, Anslyn EV, Ren P, Tsai KY, Dalby KN (2015) Quantification of a pharmacodynamic ERK end point in melanoma cell lysates: toward personalized precision medicine. ACS Med Chem Lett 6:47–52

8. Rothman DM, Shults MD, Imperiali B (2005) Chemical approaches for investigating phosphorylation in signal transduction networks. Trends Cell Biol 15:502–510

9. Shults MD, Imperiali B (2003) Versatile fluorescence probes of protein kinase activity. J Am Chem Soc 125:14248–14249

10. Shults MD, Janes KA, Lauffenburger DA, Imperiali B (2005) A multiplexed homogeneous fluorescence-based assay for protein kinase activity in cell lysates. Nat Methods 2:277–283

11. Lukovic E, Gonzalez-Vera JA, Imperiali B (2008) Recognition-domain focused chemosensors: versatile and efficient reporters of protein kinase activity. J Am Chem Soc 130:12821–12827

12. Lukovic E, Taylor EV, Imperiali B (2009) Monitoring protein kinases in cellular media with highly selective chimeric reporters. Angew Chem Int Ed 48:6828–6831

13. Stains CI, Lukovic E, Imperiali B (2011) A p38 alpha-selective chemosensor for use in unfractionated cell lysates. ACS Chem Biol 6:101–105

14. Beck JR, Peterson LB, Imperiali B, Stains CI (2014) Quantification of protein kinase enzymatic activity in unfractionated cell lysates using CSox-based sensors. Curr Protoc Chem Biol 6:135–156

15. Beck JR, Zhou XQ, Casey GR, Stains CI (2015) Design and evaluation of a real-time activity probe for focal adhesion kinase. Anal Chim Acta 897:62–68

16. Szalewski DA, Beck JR, Stains CI (2014) Design, synthesis, and evaluation of a selective chemosensor for leucine-rich repeat kinase 2. Bioorg Med Chem Lett 24:5648–5651

17. Kelly MI, Bechtel TJ, Reddy DR, Hankore ED, Beck JR, Stains CI (2015) A real-time, fluorescence-based assay for rho-associated protein kinase activity. Anal Chim Acta 891:284–290

18. Papp E, Tse JK, Ho H, Wang S, Shaw D, Lee S, Barnett J, Swinney DC, Bradshaw JM (2007) Steady state kinetics of spleen tyrosine kinase investigated by a real time fluorescence assay. Biochemistry 46:15103–15114

19. Tsang E, Giannetti AM, Shaw D, Dinh M, Tse JK, Gandhi S, Ho H, Wang S, Papp E, Bradshaw JM (2008) Molecular mechanism of the Syk activation switch. J Biol Chem 283:32650–32659

20. Jon R, Beck AL, Tung AS, Harris EN, Stains CI (2016) Interrogating endogenous protein phosphatase activity with rationally designed chemosensors. ACS Chem Biol 11 (1):284–290. doi 10.1021/acschembio.5b00506

21. Gopalakrishnan S, Harris EN (2011) In vivo liver endocytosis followed by purification of liver cells by liver perfusion. J Vis Exp 57: e3138

22. Masarone M, Federico A, Abenavoli L, Loguercio C, Persico M (2014) Non alcoholic fatty liver: epidemiology and natural history. Rev Recent Clin Trials 9:126–133

23. Peterson LB, Yaffe MB, Imperiali B (2014) Selective mitogen activated protein kinase activity sensors through the application of directionally programmable D domain motifs. Biochemistry 53:5771–5778