Studying nuclear protein import in yeast

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Accepted 15 July 2006

Abstract

The yeast Saccharomyces cerevisiae is a common model organism for biological discovery. It has become popularized primarily because it is biochemically and genetically amenable for many fundamental studies on eukaryotic cells. These features, as well as the development of a number of procedures and reagents for isolating protein complexes, and for following macromolecules in vivo, have also fueled studies on nucleo-cytoplasmic transport in yeast. One limitation of using yeast to study transport has been the absence of a reconstituted in vitro system that yields quantitative data. However, advances in microscopy and data analysis have recently enabled quantitative nuclear import studies, which, when coupled with the significant advantages of yeast, promise to yield new fundamental insights into the mechanisms of nucleo-cytoplasmic transport.

Keywords: Nuclear transport; Karyopherins; Import; Yeast; Saccharomyces cerevisiae; Quantification; Transport assay; Importin; Nucleus

1. Introduction

The hallmark feature of eukaryotic cells is the presence of a nucleus, which is defined by the nuclear envelope (NE). Operationally, the NE physically separates nuclear DNA from the cytoplasm; segregating the sites of gene transcription and ribosome biogenesis from the site of protein synthesis. This compartmentalization allows the cell to strictly coordinate numerous key cellular processes, but it also demands that an astonishing number of proteins and RNAs move between the nucleus and cytoplasm. As a result, eukaryotic cell survival is dependent upon bi-directional nucleo-cytoplasmic transport pathways. To understand the mechanisms that drive nucleo-cytoplasmic transport pathways, and how they influence cell growth, numerous studies have focused on unraveling the web of physical interactions that facilitate transport [reviewed in 1–3]. Since the late 1980s our knowledge of the molecular machinery that drives this process and the roles individual components play have increased in parallel with the development of experimental techniques that have permitted the genetic identification, biochemical purification, and visualization of these cellular factors. Collectively, these studies have revealed that, operationally, nuclear transport pathways can be divided into two phases: a stationary phase, comprised of the NE and the macromolecular protein complexes, termed nuclear pore complexes (or NPCs), that are embedded in it; and a soluble (or mobile) phase, which includes nuclear transport receptors, their regulators, and the cargoes they translocate.

NPCs are central to all nucleo-cytoplasmic exchange as they are the conduits through which all communication between the nucleoplasm and cytoplasm occurs. NPCs are highly organized, evolutionarily conserved macromolecular protein assemblies ~45–60 MDa in size, ~100 nm in diameter [4,5], and composed of ~30 distinct proteins, termed nucleoporins or Nups [6,7]. Nups can be divided into three classes [reviewed in detail in [8–11]]. Pore membrane proteins (or poms) anchor the NPC to the NE. Non-FG Nups that are thought to primarily provide the positioning scaffold for the third class of nucleoporins, the FG Nups. FG-Nups contain degenerate repeats of the dipeptide...
phenylalanine–glycine (FG) and, with a few exceptions, are symmetrically distributed on both the cytoplasmic and nuclear faces of the NPC [6,7]. These FG repeat containing Nups are thought to facilitate active transport by providing the binding sites for transport complexes traversing the NPC and, thus, directing the rapid accumulation of proteins and other macromolecules in the nucleus or cytoplasm.

Signal-mediated nuclear transport is dependent on the recognition of signal sequences present in the cargo molecule. Proteins are marked for nuclear import and nuclear export by the presence of nuclear localization signals (NLSs) [12] or nuclear export signals (NESs) [13,14], respectively. These targeting signals are recognized by soluble transport receptors termed karyopherins, or Kaps (they are also known as importins, transportins, and exportins) [reviewed in 1, reviewed in 2]. Eukaryotic cells contain two structurally related families of Kaps: the β-karyopherins (β-Kaps) and the α-karyopherins (α-Kaps). There are 14 β-Kaps in S. cerevisiae and more than 20 β-Kaps in higher eukaryotes that can be divided into three categories depending on the direction in which they transport cargo: import β-Kaps, export β-Kaps and β-Kaps that are capable of both importing and exporting cargoes [reviewed in 1, reviewed in 2]. In general, a given β-Kap recognizes and interacts directly with its cargo and facilitates the cargo’s translocation across the NE by interacting directly with FG-Nups [reviewed in 1, reviewed in 15–19]. The exception to this trend comes from the first import pathway characterized, the Kap β1/Kap α (Kap95p/Kap60p in yeast) transport pathway [20]. In this instance, the α-Kap/Kap60p acts as an adapter, bridging the interaction between the cargo and Kap β1/Kap95p, which mediates the movement of this trimeric import complex through the NPC.

The small GTPase Ran is also a key regulator of transport that provides directionality to nucleo-cytoplasmic transport, in part by regulating the formation of Kap-cargo complexes [21,31–42]. In metazoan cells, Ran cycles between a GTP- and GDP-bound state. The cytoplasmic localization of RanGTPase-activating protein [25,26] and the nuclear localization of it’s guanosine nucleotide exchange factor (RanGEF) [27,28], generates a gradient of RanGTP across the NE. This gradient is thought to control the association and dissociation of Kap-cargo complexes [24,29]. Accordingly, import complexes form in the cytoplasm where the concentration of RanGTP is low. Once on the nuclearplasmic face of the NPC, import complexes encounter an environment rich in RanGTP. Here, RanGTP binds the import β-Kap, stimulating Kap-cargo complex dissociation and terminating the import cycle. Conversely, export karyopherins bind their cargoes cooperatively with Ran-GTP in the nucleus. These nuclear export complexes dissociate once they reach the cytoplasm, where RanGAP induces GTP hydrolysis [reviewed in 2,10,11, reviewed in 18,23,24].

Now that the roles of many of the soluble transport factors have been defined, the remaining questions about nucleo-cytoplasmic transport are rather more subtle, concerning how exactly individual transport reactions proceed, how they determine transport rates and how they are regulated. Biochemical methods have generated much useful data using purified proteins with in vitro solution binding assays [reviewed in 8–11]. Hypotheses based on these biochemical findings have been tested in in vitro nuclear import assays. These assays permeabilize the plasma membrane of tissue culture cells with digitonin [30], then re-supply the system with purified transport factors to reconstitute nucleo-cytoplasmic transport in a semi-cell-free environment. Using this technique, a number of the key soluble cellular factors and metabolites required for nuclear transport in vitro were characterized [21,31–42]. This in vitro system has also been used to measure the import rates of transport factors with or without their cargoes [43,44], and has been coupled with single molecule microscopy methods to measure the millisecond dwell times of NTF2 [45] and Kap-cargo import complexes at the NPC [44]. In vitro techniques have also been employed to study import in Xenopus oocytes, either using intact nuclei [46] or by adhering nuclear envelopes over microscopic wells, such that import into these wells recorded the transport of single NPCs [47–49]. These studies have provided information about the rates of individual transport reactions, but it is unclear whether these transport rates relate to the situation in vivo, where the import of a particular cargo likely competes with that of many other cargoes and transport pathways. Microinjection of labeled cargoes into intact tissue culture cells [48,95,94] or Xenopus oocytes [98] have thus provided more physiologically relevant import rates, that often differ from their in vitro counterparts.

While the techniques described above were developed to study nucleo-cytoplasmic transport in metazoan cells, S. cerevisiae is the most genetically tractable nucleated model organism, in which it is possible to make systematic alterations to components of its nuclear translocation machinery. Many of the techniques available to perform such manipulations in yeast are either completely unavailable or prohibitively time-consuming in metazoan cells. In yeast, proteins of interest can be expressed from either genomic integrations, either using intact nuclei [46] or by adhering nuclear envelopes over microscopic wells, such that import into these wells recorded the transport of single NPCs [47–49]. These studies have provided information about the rates of individual transport reactions, but it is unclear whether these transport rates relate to the situation in vivo, where the import of a particular cargo likely competes with that of many other cargoes and transport pathways. Microinjection of labeled cargoes into intact tissue culture cells [48,95,94] or Xenopus oocytes [98] have thus provided more physiologically relevant import rates, that often differ from their in vitro counterparts.
adapt the in vitro nuclear import techniques previously described for metazoan cells to yeast cells have been only mildly successful [101]. Furthermore, the study of nucleo-cytoplasmic transport of cargoes in an in vivo context can often be complicated by the biological processes that these cargoes are involved in and affected by. The recent development of alternative nuclear import assays has addressed many of these problems and, as a result, has opened the door to quantitatively studying nucleo-cytoplasmic transport in the most genetically tractable model organism.

Here, we will first describe traditional methods used to study nucleo-cytoplasmic transport of full-length fluorescent fusion proteins in vivo. We will also highlight and focus on a technique that uses galactose-inducible expression of fluorescent cargoes to characterize the molecular mechanisms that mediate the nuclear import of cellular factors that are bound and retained in the nucleus after transport [51]. To further study cargo-import, independent from other biological processes involving the cargoes, we provide an outline of how to define nuclear localization signals within proteins of interest. We then present three methodologies that can be used to examine the import rates of fluorescent reporter proteins bearing such NLSs. Each of these methods requires a different investment of effort to return measurements of an increasingly quantitative nature. In the first case, relative import rates can be obtained from measurements of the NLS-GFP protein’s steady-state nuclear versus cytoplasmic concentration ratio, measured directly from growing cells. The second technique can measure more subtle changes in import by using a variety of metabolic energy poisons to inhibit receptor-mediated nuclear transport; this treatment prevents the formation of RanGTP, which is essential to most signal-mediated import. Re-import of the cargo is then studied in the population of treated cells, after they have been relieved of this transport-poison, thus providing relative measurements of fluorescent cargo import rates [52]. The final technique is a high-resolution quantitative single cell assay, whose central methodology is directly based on this poison-based assay, but involves substantial modifications to provide necessary quantitative information to measure the import efficiency of the pathway(s) importing a fluorescent model cargo (Timney et al., manuscript in preparation).

2. Overview

The simplest measurement of import or export of a cargo is to monitor its accumulation in the nucleus (N) relative to the cytoplasm (C) or vice versa—typically expressed as an N/C ratio. For example, when defining the β-Kap that mediates the nuclear import of the cargo protein of interest a fusion protein containing the cargo and a reporter protein is expressed in wild-type and mutant yeast cells. If the mutant strain encodes a protein that is required for, or enhances, the nuclear import of the cargo, then it will be imported at a rate different from that observed in wild-type cells. As a result, the respective N/C distributions observed in these strains will proportionally reflect these import-rate differences. Fluorescence microscopy can be used to monitor these changes in subcellular localization.

Indirect immunofluorescence and in vivo fluorescence microscopy techniques could be used to accomplish this task, of monitoring a protein’s subcellular distribution. However, in vivo studies are simpler and allow researchers to obtain rapid and direct results from complex experiments, which can include extended time course experiments, multiple treatment conditions, or both. As a result, in addition to being able to rapidly study numerous constitutive nuclear transport events, it is possible to study the dynamic aspects of the system. In vivo transport assays have also paved the way for single cell studies.

2.1. Yeast mutant strains

The nuclear transport assays described here will work equally well regardless of whether yeast knockout or temperature-sensitive mutants strains are used. Deletion or temperature-sensitive mutant strains have been constructed for virtually all yeast Nups, as well as the β-Kaps, yeast Ran (Gsp1p) and its regulators, providing an almost complete repertoire of mutant strains for all known yeast nuclear transport factors. Although most of the central players in nucleo-cytoplasmic transport have been identified, the generation of yeast deletion and titratable promoter strain libraries has made it possible to rapidly screen for additional factors involved in nucleo-cytoplasmic transport, the list of which is constantly growing [53,54]. The Saccharomyces Genome Deletion Project contains a set of yeast deletions strains, in which >95% of all yeast ORFs in the Saccharomyces Genome Database were disrupted [55]. In addition, Mnaimneh et al. [56] recently constructed a library of promoter-shutoff promoters for over two-thirds of all essential yeast genes. This collection includes 800 strains, each with a kanR-tetO2-TATA cassette integrated in place of the endogenous promoter driving the expression of an essential yeast gene. Thus, the expression of these genes can be turned off when the cells are cultured in the presence of doxycycline, allowing for the down regulation of the promoter until the gene of interest is no longer expressed at detectable levels [56]. Both the Yeast Genome Deletion collection and the Tet-promoter library are commercially available from Open Biosystems (Huntsville, AL).

2.2. Construction of fusion proteins

The ability of these imaging experiments to reveal a connection between a nuclear transport factor (Nup, Kap, or transport cofactor) and the transport pathway or individual cargo in question is dependent on the experimenter’s ability to visualize a change in the subcellular localization of a fluorescent reporter protein. These proteins are typically constructed by fusing the gene encoding the protein in question in frame at its 3’ end to a cassette encoding a
fluorescent tag and a selectable marker. The resulting recombinant DNA cassettes can then be expressed either exogenously from an autonomously replicating expression vector or incorporated directly into the genome of the strain(s) of interest via homologous recombination. Expression of these FP gene chimeras can be optimized for fluorescence microscopy analysis by placing them under the control of different regulatory elements [50]. For example, genomic integration of the FP chimeric gene or a combination of CEN vectors and endogenous regulatory elements can be used to closely replicate endogenous expression levels, while constitutive promoter elements (i.e., ADH1, PGK1, TP1I or PRK1) can be used to increase synthesis of the protein chimera to optimal levels for visualization. Alternatively, inducible promoter elements (i.e., GALI or CUP1) can be used to specifically control expression of the recombinant gene, thus allowing their expression to be turned on and off when desired [51,57]. However, it is important that initial experiments are performed under conditions where endogenous expression levels are replicated as closely as possible because altering the native expression patterns of any cellular factor could produce an artificial environment that is not physiologically relevant. Regardless of the expression system used, all constructs should be rigorously tested to ensure that the fusion proteins behave in the same manner as the untagged target protein.

While the brightness of an FP fusion protein is primarily dependent on its expression level, the intrinsic brightness of the FP tag, as well as the folding and translation efficiency of the FP also influence its overall brightness and visibility. Folding efficiency of the FP is primarily dependent on environmental conditions [58,59], but it can also be influenced by the target protein. Therefore, bridging the intersection between the target protein and the FP with a short linker peptide has been shown to enhance the maturation of the fusion protein [59]. In addition, translation efficiency is affected by codon usage, therefore researchers have constructed yeast-optimized FPs to improve the overall expression of these tags in yeast [60,61]. Moreover, for imaging experiments that require long exposures, one must also consider the rate at which the FP photobleaches [59].

GFP is the most commonly used fluorophore for the experiments described here, but a number of GFP variants are now available, including yeGFP [60], EGFP, EBFP, ECFP, EYFP (Clontech, Mountain View, CA), DsRed [62], hcRED [63], and RedStar [64]. Janke et al. recently constructed a new toolbox of tagging vectors [65], which includes 37 new cassettes for C-terminal epitope tagging using a variety of different fluorescent proteins in all colors available, new selection markers, and a variety of epitope tags. There are also 37 N-terminal tagging cassettes, which not only facilitate the N-terminal tagging of the target but also allow the replacement of the endogenous promoter with an inducible one. Each of these GFP derivatives has been successfully used in yeast and can theoretically be used to monitor the localization of any yeast protein. The suitability of each FP must be tested for each target protein being analyzed.

2.3. Hints for troubleshooting

It is important to note that for most experiments, the absolute brightness of the FP does not appear to be nearly as important as reducing or combating the inherent autofluorescence of yeast cells. Using an FP that fluoresces in a region of the spectrum with low yeast autofluorescence, even if the FP is less bright than others, is often advantageous. But, this may not always be possible as the autofluorescent spectra are generally broad and overlap with the emission wavelengths of GFP and many of its derivatives. Many cellular components, as well as components of the culturing medium, exhibit autofluorescence [58]. It is therefore important that the filter sets used in these experiments are closely tailored to the targeted emission spectrum. Various software tools can also be used to remove the autofluorescence signal and enhance FP visualization in the resulting images. Another point to consider is that some yeast strains also produce more autofluorescent metabolites than others. For instance, the W303 strain can be considerably more autofluorescent than strains such as DF5, particularly when approaching stationary growth or when grown on plates. Finally, studies have demonstrated that some yeast media, such as yeast extract, emits a broadband fluorescence that may combine with poor expression and weak emission of the FP to completely mask its signal [58]. Therefore, for the imaging experiments described here, yeast cells are grown in complete synthetic media (CSM—0.17% YNB-AA/AS, 0.5% (NH4)2SO4, CSM powder according to the manufacturer (Difco)) supplemented with glucose or an alternative carbon source. While commercially available yeast nitrogen base is most commonly used, some researchers have found that it can also be highly fluorescent and prepare custom low fluorescence YNB [66].

3. Subcellular localization studies of full-length import-cargoes in yeast

3.1. Microscopy

In vivo fluorescence localization studies and changes in the steady-state distributions of FP chimeras and nuclear transport factors as a result of a genetic or environmental perturbation are often sufficient to qualitatively evaluate nucleo-cytoplasmic exchange. We have used both laser-scanning confocal microscopy (LSM 510 NLO; Carl Zeiss, Inc.) and conventional microscopy (Axioskop2 or Axiophot; Carl Zeiss, Inc.), and have found that both systems work well. Oil immersion 63× and 100× lenses (numerical apertures = 1.4 or 1.25, respectively) have typically been
3.2. Subcellular localization assays using constitutively or endogenously expressed FP fusion proteins

Initially wild-type strains are used to determine the natural cellular localization of the cargo-protein in question and to define the cellular and/or environmental conditions that normally lead to its nuclear transport. These initial experiments should address whether the protein of interest constitutively localizes to the nucleus or cytoplasm with an apparently steady-state distribution, or transiently moves between these two compartments of the cell. When transient import or export events are being studied, the conditions that induce translocation should also be defined. Finally, when monitoring nuclear import, it is also important to establish whether the cargo is also actively exported from the nucleus and determine which transport, binding or processing events are most important in determining the steady-state or fluctuating cellular localization(s) of that protein. Once these conditions have been defined, simple side-by-side visual comparison of the images obtained from wild-type and mutant yeast strains, either knockout or ts mutants (Fig. 1), should reveal whether the nuclear transport factor encoded by the mutant allele is required to maintain the wild-type cellular distribution of the protein of interest, be it a cargo molecule, karyopherin or soluble transport cofactor.

When ts mutants are being studied, starting cultures of the parental and mutant strains are grown at 23 °C and the subcellular localization of the FP fusion protein determined at the permissive temperature. Typically, at this point in the experiment, the localization of the protein of interest should be the same in both strains (Fig. 1). The cultures are then shifted to the non-permissive temperature (37 °C) and, typically, re-examined after the shift. Cultures are imaged periodically over several hours. The extent to which a cargo mislocalizes varies, and is unique to each cargo and strain being studied (see Fig. 4C). These results may also vary from experiment to experiment depending on factors such as culture density. Therefore, even subtle changes in localization patterns can be indicative of a functional role in cargo movement and additional experiments should be performed to precisely define its requirement.

3.3. Inducible nuclear import assays

The success of the subcellular localization assays on mutant strains described above are occasionally dependent on whether the biology of a given cargo allows its distribution to perceptibly change, during the time course of such an experiment. For instance, a protein that is not actively shuttled into and out of the nucleus may not change its distribution rapidly in response to a mutation in even its cognate Kap. Also, many proteins are imported and localized to distinct subnuclear domains, like the nucleolus, immediately following translocation and this remains their final destination [51]. In these cases, it becomes difficult to characterize the nuclear transport pathway that mediates the import of such cargoes, as they fail to accumulate in the cytoplasm upon β-Kap inactivation; this may be particularly problematic for some ts strains that can often only kept viable for a few hours at restrictive temperatures. Given that most nuclear import cargoes display some degree of pleiotropic mislocalization under these conditions, one could be convinced that another member of the β-karyopherin protein family is importing these cargoes. However, it is also possible the alternative mechanisms such as binding and retention within a subnuclear domain tether these cargoes in the nucleus. To distinguish between these two possibilities, Leslie et al. [51] developed an inducible in vivo fluorescence nuclear import assay. This modification was successfully used to identify the transport receptor that mediates the import of numerous nucleolar proteins whose post-import nuclear retention made it technically impossible to characterize these transport mechanisms using conventional nuclear import assays (Fig. 2) [51].
This assay is based on the assumption that if the insoluble nuclear protein being studied is a \textit{bona fide} import cargo of the β-Kap in question, then inducing its expression after β-Kap inactivation will reveal whether this transport pathway is required for its nuclear import. Furthermore, while this assay has only been used when studying conditional yeast mutant strains, it could also be used to define primary and secondary transport pathways when multiple β-Kaps transport one particular cargo protein. To this end, cargo-FP fusion genes are placed under the control of an inducible promoter (e.g., \textit{GAL1} promoter in the yeast 2 \mu m expression vector pYES2; Invitrogen, Carlsbad, CA) transformed into the strains of interest and cultured at 23 °C under non-inducing conditions (i.e., in media containing glucose or raffinose as a carbon source). To inactivate the temperature-sensitive β-Kap of interest, the cultures are then divided in half and incubated at permissive and restrictive temperatures (e.g., 23 or 37 °C for 2h). Following inactivation, cells from each culture are quickly harvested via centrifugation, washed with sterile water and resuspended in preheated induction medium (e.g., CSM supplemented with 2% galactose) and incubated at 23 or 37 °C for an additional 2–3 h to induce expression of the cargo-FP gene fusions. The subcellular localization of the FP fusion protein is then established using direct fluorescence microscopy (Section 3.1). If the insoluble nuclear protein is transported by the β-Kap in question then it should accumulate in the cytoplasm of the mutant cells when its expression is induced at 37 °C, but be transported to its appropriate nuclear or subnuclear domain at 23 °C or when a wild-type copy of the transport receptor is present (Fig. 2).

3.4. Controls

Before one can conclude that the transport factor being studied (be it a Nup, β-Kap or some other accessory factor), is required for a nuclear transport event to proceed in an efficient manner a number of controls must be performed. In addition to parallel experiments being carried out in parental and mutant strains, the localization of the FP fusion protein should also be assessed after a functional copy of the ts or knocked-out allele has been added back to the mutant strain. If multiple ts strains are available, the localization of the fusion should be monitored in each of these strains. This type of control experiment can demonstrate whether the subcellular localization of the protein of interest is affected by allele or strain-specific variations [67,68]. Controls assessing the specificity of the transport block being studied should also be performed. To this end, the cellular localization of a cargo molecule that is not transported by the import or export pathway being studied should also be monitored.

3.5. Hints for troubleshooting

Many ts alleles are naturally sensitive to temperature fluctuations that could potentially affect the outcome of an experiment, even if those changes are slight. Hence, water bath incubators are preferable for ts cell growth, as they are less susceptible to temperature fluctuations than air incubators. Moreover, it is also important that the temperature of the cultures be maintained throughout the imaging step of the experiment. We have found that using a heated microscope stage helps maintain the desired culture temperature. It should also be noted that growth at 37 °C is stressful for yeast cells. The heat shock response pathway is activated to circumvent many of the secondary instabilities associated with growth at elevated temperatures. As a result, the targeting of many macromolecules to the nucleus ceases under these conditions [69–73], while the import of some proteins is facilitated by heat shock protein expres-
sion [52]. Moreover, ts mutants are by nature unhealthy and often grow poorly even at permissive temperatures. It is advised that the cells be evaluated at a range of temperatures, paying particular attention to the morphology of the nucleus and the ability of cells to recover following the shift.

3.6. Limitations

As mentioned above, several members of the β-karyopherin protein family can recognize and import common cargoes [51,74–79]. These redundancies, which are collectively referred to as nucleo-cytoplasmic transport networks, add an additional level of complexity to the characterization of Kap-cargo interactions and complicate the interpretation of the data acquired from the localization studies described here. These imaging experiments simply answer the question: Does a particular perturbation alter the subcellular localization of the protein in question relative to that observed in wild-type cells? Therefore, they are most easily recognized by the putative cargo molecule independent of the other functional domains of that protein.

4.1. Defining candidate NLSs

NLSs and NESs are typically short peptides that do not fit a well-defined consensus sequence and can be found anywhere within the sequence of a protein. Nevertheless, studies have demonstrated that these signals are generally recognized by specific Kaps [see examples in 1,81]. The lack of a simple, reliable primary sequence consensus has made it difficult to predict which amino acids will be required for transport. As a result, the most straightforward way to identify these signal sequences is to construct a series of fragments of the original cargo molecule as FP fusions and monitor their subcellular localizations in vivo, essentially as previously described (Section 3). We focus from this point on the identification of NLSs, although complementary methods could be adapted to identify NESs.

A less than random process for the generation of putative NLS-FP fusion proteins can be adopted if there is a known or putative β-Kap for the import of the cargo in question. By applying the generalities in basicity and hydrophobicity of NLS sequences recognized by the putative β-Kap, the number of cargo deletion fragments to be made can be reduced, and the truncation of potential nucleophilic sequences avoided. Amino acid sequence alignment software packages, such as MegAlign (DNASTar, Inc., Madison, WI) and ClustalW (http://www.ebi.ac.uk/clustalw, European Bioinformatics institute, Cambridge, UK), can be used to identify regions that share some degree of identity or similarity with previously characterized NLSs recognized by the β-Kap [51,82,83]. For example, when characterizing the NLS of Sof1, the full-length amino acid sequence of Sof1p was independently aligned with each of the previously identified lysine-rich NLSs of the putative β-Kap for this cargo, Kap121p, (Table 1) using MegAlign (Lipman-Pearson: kputle, 2; gap penalty, 4; gap length penalty, 12). Random sequence similarities and aligned NLS sequences avoid the signiﬁcance of the role that transport factor plays in the import of that cargo molecule. For example, loss of function of the primary transport receptor of a cargo should result in a “strong” cargo mislocalization phenotype, while loss of a secondary transporter is more likely to result in a more subtle mislocalization phenotype [74,75,80]. This type of analysis can be used to assign a significance order or rank to each transport pathway importing the common cargo. These hypotheses can then be tested using the semi-quantitative and quantitative microscopy methodologies described below.

4. Defining nuclear localization signals

The observed subcellular distributions of full-length cargo proteins, as discussed in the previous section, are frequently complicated by subsequent cellular processes in which those cargoes may be involved. This is a natural consequence of examining functional proteins in their in vivo environment. Ribosomal proteins are a good example of the complexity of the combined interactions that can give rise to the steady-state distribution of a full-length cargo. Ribosomal proteins are translated in the cytoplasm, imported into the nucleus and assembled into ribosomal subunit precursors in the nucleolus. These subunits are then exported back to the cytoplasm where they function [reviewed in 102]. Thus, the steady-state distribution of each ribosomal protein is a complicated combination of cytoplasmic, nuclear and nucleolar localizations. Therefore, it is difficult to study the import or export of a ribosomal protein by examining its steady-state distribution as a full-length ribosomal protein-FP in wild-type or mutant strains. Experiments like those described in the previous section, using galactose-inducible expression of cargo molecules, can in some cases be used to overcome these technical difficulties. However, it is often necessary, and informative, to identify the NLS or NES-containing regions of the cargo molecule. Once identified, NLS-FP or NES-FP fusion proteins can be used to characterize the import or export pathway that is required for the nuclear import of the full-length protein independent of the other functional domains of that protein.
The NLS sequences present of previously characterized Kap121p nuclear import cargoes are presented here. These amino acid residue sequences are written using the standard single letter abbreviations.

<table>
<thead>
<tr>
<th>Cargo</th>
<th>Amino acid residues</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pho4p</td>
<td>140–166</td>
<td>SANKVTKNKSSNPSPYLNKRRKGKPGDS</td>
<td>[96]</td>
</tr>
<tr>
<td>Spo12p</td>
<td>76–143</td>
<td>KKTSNLKSSHTTLSVVKTMKDRLQLQDPRKLQLQFASPTDLVPCSLKLNEHKVK</td>
<td>[85]</td>
</tr>
<tr>
<td>Pdr1p</td>
<td>729–769</td>
<td>WTDNMKILLLDNDVSYRSFAHYSICILVSYSAV</td>
<td>[97]</td>
</tr>
<tr>
<td>Rpl25p</td>
<td>1–62</td>
<td>MAPSATAAKAVKGTNGKALKVRTSATFRLPKTLKARAPIKYPASKAVPHYNRLDSY</td>
<td>[77]</td>
</tr>
<tr>
<td>Yalp1p</td>
<td>5–59</td>
<td>TAKRLSDVVSGLSLEEFSGSKRSDIEJNEHHRGTGRDGDSEQPKKGKSKTKKK</td>
<td>[80]</td>
</tr>
<tr>
<td>Histone H2A</td>
<td>1–46</td>
<td>MSGKGGKAGSAAKASAQAKAGLTFPVGRVRHSLRGRNYAQRIG</td>
<td>[75]</td>
</tr>
<tr>
<td>Histone H2B</td>
<td>1–52</td>
<td>MSAAEKKPKSAPAAEKPKAAPKTSDDGKVRKSKARTEYSSYIYVKLTQT</td>
<td>[75]</td>
</tr>
<tr>
<td>Histone H3</td>
<td>1–28</td>
<td>MARTKQTFARKSTGGKAPKRGKLQASKAARK</td>
<td>[74]</td>
</tr>
<tr>
<td>Histone H4</td>
<td>1–42</td>
<td>MGRGKGKGKGLKGKAKRHKLRAQDQSITGAPAIRLARRGG</td>
<td>[74]</td>
</tr>
<tr>
<td>Aft1p</td>
<td>198–226 and 332–365</td>
<td>TSSIKPIKCRKCSRFNCPFVRATYSL and SKRPCLPSVNTGSINTNNVRKPGKSCQCNKDVL</td>
<td>[86]</td>
</tr>
<tr>
<td>Ste12p</td>
<td>494–688</td>
<td>NNMLYPTATSWNVLPPAMQAPTPYVTGRPYTPNYLRPGSAMPFYMPSQSNMOWNTAVSP</td>
<td>[82]</td>
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<tr>
<td>Nop1p</td>
<td>1–90</td>
<td>MGRPGRGSRGGRGSGGGRGGRGGRGGRGGRGGRGGRGGRGGRGGRGGRGGRGGRGGRGGRGGRGGRGGRG</td>
<td>[51]</td>
</tr>
<tr>
<td>Sof1p</td>
<td>381–489</td>
<td>ENRSNVTTRKLNKLEYDKLKERFRHMEPKRSRHVRHPVEQKIAKEQIKNLSSIKREAN</td>
<td>[51]</td>
</tr>
</tbody>
</table>

The NLS sequences present of previously characterized Kap121p nuclear import cargoes are presented here. These amino acid residue sequences are written using the standard single letter abbreviations.
5. Import rates of NLS-GFP cargoes can be estimated from their N/C ratios in growing cells

NLS-GFP reporter proteins as those defined above are used as models to study the nuclear import of many cargo molecules. These model-cargoes are free of many of the other functional domains of a protein that may otherwise strongly bind to target sites (such as for Sof1p) or cause it to be processed or regulated in ways that can confound the analysis of its import as a full-length protein (such as for Rpl25p). Second, full-length FP fusions are often significantly larger than the ~40 kDa diffusion limit of the NPC, whereas NLS-GFP constructs are typically smaller than this. This means that NLS-GFP constructs can leak out of the nucleus within relatively short time-scales of import being perturbed (say by a specific ts mutation), whereas a full-length protein larger than 40 kDa may be unable to redistribute during a physiologically useful time scale [87]. For these model-cargoes, the simplest evaluation of their import rates derives simply from a measurement of their

Fig. 3. Defining nuclear localization signals (NLSs). (A) Schematic diagram of Sof1p. The NLS sequences of previously characterized Kap121p nuclear import substrates (Table 1) were compared to full-length Sof1p by using MegAlign (Lipman-Pearson: ktuple, 2; gap penalty, 4; gap length penalty, 12). (B) Galactose-inducible GFP chimeric proteins containing the four fragments of Sof1p were synthesized at 23 °C in kap121-34 cells and analyzed by direct fluorescence microscopy. Note the nuclear localization of Sof1p(aa381–489)-GFP and Sof1p(aa411–450)-GFP (images originally published in 51). (C) NLSs were identified in several proteins that bound Kap123p in biochemical assays, and were compared with the import of the NLS of the previously described Kap123p cargo Rpl25p [77]. Potential NLSs were located by visual inspection of each protein’s sequence, compared with the proposed ribosomal NLS consensus [84]. Potential NLS sequences (Table 2) were then expressed as GFP fusion proteins from 2 μ plasmids under the TPI1 promoter. Images were collected using spinning disk confocal microscopy, from which serial image sections have been combined as a maximum projection. These domains were defined as putative NLS if they localized to the nucleus in wild-type cells and if that localization was sensitive to metabolic energy poisons (not shown) (images are from Timney et al., manuscript in preparation).
accumulation in the nucleus (N), relative to the cytoplasm (C)—typically expressed as an N/C ratio.

NLS-GFP cargoes are imported by their cognate Kap (or Kaps) at a particular rate, but are also small enough to leak back out of the nucleus by simple diffusion through NPCs. Thus, cells in culture have a distribution of these NLS-GFP fusion-proteins that has reached a steady-state, with their rate of NLS-GFP import matching the rate of NLS-GFP leak out of the nucleus. It can be shown that if import of cargo is a kinetically first order process, with rate constant $k$, and the leak of naked cargo is through passive diffusion, with NPC permeability constant $p$, then this N/C ratio corresponds simply to the relationship $k/p + 1$ [87]. If a cargo is not actively imported, $k$ is 0, therefore its steady-state N/C ratio will be 1 (i.e., evenly distributed), while two cargoes with similar passive NPC permeabilities (i.e., cargoes of similar sizes) that are imported at different rates will proportionally reflect these rates in their respective N/C values. Since subcellular fluorescence is proportional to the concentration of the fluorophore, the measurement of N/C ratio of NLS-GFP cargoes can be made directly from background-subtracted microscopy images; cells are simply grown to logarithmic growth phase and imaged (see Sections 3.1 and 6.3 for descriptions of how such images can be acquired). Most imaging software provides tools to measure the pixel intensities within selected regions of interest, that can be created either by hand, or using software specific tools such as thresholding or edge detection algorithms. It is possible to measure cellular N/C ratios from microscopy images using a variety of software packages, including but not limited to, Metamorph (Universal Imaging Corporation), Imaris (Bitplane AG), Openlab (Improvision) and the custom software developed using the Image Analysis Toolpack of MatLab (The Mathworks). Each work equally well at capturing pixel intensities of nuclei and cytoplasmic, but vary in their subjective ease of use and their available tools, processing functions and automatability. This last point is often important, since measurements of N/C ratio need to be taken from many cells in a population to provide statistically reliable measurements.

This ratio is simple to derive, and can be obtained from cells that have not been treated with any potentially harmful chemicals. Furthermore, it is underappreciated that this cargo N/C ratio can provide researchers with important information about the relative import rates of their cargoes, simply by comparing these ratios between different strains. For example, a 5-fold decrease in the N/C ratio of a given NLS-GFP cargo, resulting from the addition of a particular β-Kap, probably indicates a 5-fold decrease in the import-rate of that cargo. But, the relationship that determines the steady-state N/C ratio of a cargo also includes NPC permeability constant, and can be used for relative comparisons only where cargo permeabilities are different. We would be unable to distinguish whether a change in cargo N/C ratio had resulted from disparate import capacities of the altered NPCs, or from a change in the leakiness of these pores. Similarly, the absence of a detectable change in N/C ratio could either indicate that there was no functional difference between strains or that there were equal changes to both the active import and passive permeability components of accumulation. Thus, N/C ratio can only be used as a quantitative import measure when the passive permeability of a cargo is already known, and can be used for relative comparisons only where cargo permeability can be assumed to be constant between conditions.

6. Metabolic-poisoning import-assays

6.1. Rationale

To measure active import, separate from passive diffusion, measurements of nuclear accumulation of cargo must be taken when the system is far from these steady-state N/C ratios. As discussed earlier, various kinetic assays have been developed to monitor nuclear transport in particular metazoan cell types. Yeast cells are, however, much smaller than mammalian tissue culture cells or Xenopus oocytes. In addition, they have a tough cell wall. These obstacles have meant that the techniques used to measure nucleo-cytoplasmic transport in metazoan cells could not be readily adapted for use with yeast. A significant advance in measuring nuclear transport kinetics in yeast was made by Goldfarb and colleagues, who introduced a method that uses metabolic energy poisons to temporarily stop import of a NLS-GFP cargo, by destroying the Ran gradient [52]. Poison-treated yeast
Cells revive and restart transport shortly after the poison mixture is removed; import can then be quantified by scoring the percentage of cells that, over time, have visually recovered a significant nuclear signal of the cargo (Fig. 4). This technique has been successfully used to monitor the relative effect upon import of genetic alterations that would have been infeasible in other organisms. Such studies have examined deletions of structurally important Nups [87] and FG-domains [89], and have also looked for fluctuations in import during phases of the cell cycle [88].

Facilitated by modern microscopy and data processing techniques, existing yeast nuclear import assays [52] have been modified, introducing methods for rapid, semi-automated cell-by-cell quantitation of import rates. The modified method provides more sensitive detection of subtle perturbations to the import machinery and allows for quantitative import-rate measurements to be made in single yeast cell. As in the original method, transport is temporarily stopped with metabolic energy poisons, which destroy the RanGTP/GDP gradient [91]; re-import of NLS-GFP cargo is measured after these poisons are removed (Fig. 6).

Fig. 4. Overview of nuclear import assay. Illustration of import in cells treated with metabolic energy poisons for an import assay. For explanation see figure annotations.
Two key improvements were made to data collection and analysis so that import rates in single cells could be quantified and represented as units of cargo molecules/NPC/s. First, advanced automated microscopy was employed to observe import in single cells over time, while 3-D reconstruction of confocal images was used to measure cellular morphometry. Second, quantitative Western blotting was used to calibrate fluorescence measurements to subcellular concentration of the NLS-GFP cargo. Import measurements are thus calibrated for cargo concentration and normalized for cell-to-cell variations in intracellular volume and NPC number. Because measurements of nuclear accumulation of cargo are taken when the N/C ratio is almost 1, net passive flux of cargo is close to zero, therefore these initial accumulation rates are a direct measure of active import.

6.2. Plasmids and strains

NLS-GFP model cargoes are expressed constitutively under the TPI1 promoter in the 2μ multi-copy pYX242 shuttle plasmid (Novagen discontinued). Alternatively, any constitutive expression plasmid could be substituted. For these assays the following are necessary: a strain with fluorescently tagged proteins that would mark both the nuclear and cytoplasmic volumes for measurement; and a labeled protein of known abundance, to calibrate the abundance of the NLS-GFP cargoes to this internal standard. Therefore, import assays are performed in a strain background containing a Fluorescent Protein (CFP)-tagged Htb2p (a nuclear histone protein) and Tpi1p (a ubiquitous glycolysis enzyme), which mark the nucleus and cytoplasm, respectively, while Htb2-CFPp also serves as an internal calibrant for NLS-GFP concentration.

6.3. Microscopy

Images are collected at room temperature (air conditioned to 23 °C) with a spinning disk confocal microscope [for e.g., Hamamatsu Orca ER cooled CCD camera attached to a Zeiss Axiovert 200 microscope fitted with a Perkin-Elmer UltraView spinning disk confocal imaging head and using a 100× objective lens (NA 1.5)]. CFP and GFP are excited with HeCd 442 nm or Argon 514 nm lasers, respectively, and separate images of each are obtained using a standard CFP/YFP dichroic with separate excitation/emission filter sets (Chroma). These CFP/YFP optics suffice to image NLS-GFP along with Htb2-CFPp and Tpi1-CFPp with no bleed through of fluorescence seen between GFP and CFP image channels. The system described above is controlled with MetaMorph imaging software (Universal Imaging Corporation). Exposure settings are chosen during test runs, to maximize use of the 12 bit gray scale of the detector, without saturation, and these settings should be maintained for all subsequent images of the strain. Control images of a blank slide without laser excitation are taken for background subtraction of detector noise. Areas around the edge of an image field can be considerably less bright than those in the center, but this can be compensated for by acquiring a control image of a fluorescent glass slide (Chroma). This control image is normalized, so that its maximum pixel intensity is set to a value of 1. Thus, if a peripheral area of the field is 60% as bright as the center then the normalized control image would have values of ~0.6 in this area; dividing all images of cell fields by this control image will hence provide the appropriate compensation for such uneven field illumination. Some microscopy setups, such as Applied Precisions DeltaVision systems, routinely perform this image correction automatically.

6.4. Metabolic energy poisons temporarily halt transport

Yeast cells are typically grown at 30 °C in 15 mL cultures to mid-log phase in selective minimal media. For each import assay a 1 mL sample of cell culture is harvested and frozen for later NLS-GFP concentration calibration (see Section 6.8). Poison treatment and release is performed essentially as described in the original method [52]. Cells are washed with water and resuspended in 500 μL of the transport poison (SC media without a carbon source, containing 10 mM 2-deoxyglucose and 10 mM sodium azide). With transport stopped, after 15 min at room temperature the NLS-GFP cargoes will have equilibrated between the nucleus and cytoplasm—alternative methods of slowing transport, such as using only deoxyglucose or cold treating the cells [87], can be used, but are not always effective in fully equilibrating all import cargoes (see Section 6.11 for discussion). Over the course of the next hour samples are taken from this pool of poisoned cells for import assays. After that hour the poisoned cells are discarded and a new batch prepared. Despite this precaution import rates do not appear to change even after up to 75 min of incubation in the poison.

6.5. Original poison assay data collection and analysis

In the original application of this method, a sample of poisoned cells of 100–200 μL are washed several times with water, then the cells resuspended in a similar volume of SC media containing glucose, to restart import of the NLS-GFP. From this pool of released cells, 3 μL samples are taken at 5 min intervals for image acquisition using standard epifluorescence microscopy, as described in Section 3.1. At least 30 cells from each time-point are counted, with a particular cell scored as having a nuclear signal if its nucleus is brighter than the surrounding cytoplasm with a clear nuclear to cytoplasmic boundary. The percentages of cells at each time-point so judged to be “nuclear” are then plotted (Fig. 5), and the relative rates of nuclear import are calculated from linear regression lines drawn through the linear portions of the import time course.
These measurements of import can thus show relative changes in import rates between strains on a population level, such as is illustrated in Fig. 5 as an example of the other important studies over the past 10 years successfully using this assay [87–89]. However, the values obtained using this technique are not actual import rates such as those that can be obtained from single metazoan cells, where importing cargo molecules are counted. Recently though, this assay has been modified in order to provide quantitative measurements of cargo-import over time in single-cells (Timney et al., 2006, manuscript in preparation). While the original analytical method is more rapid and will still be of use in cases where relative, yet rapid, measurements are acceptable, these modifications can now provide quantitative data about comparatively subtle changes in import rates.

For the modified import assay the pool of poisoned cells are prepared as in Section 6.4 and each quantitative import assay is performed as follows.

6.6. Automated microscopy visualizes import in single cells

For each import assay, a 50–100 μL sample of the poisoned cells is harvested, washed once with water, resuspended in 10–20 μL of water to give an appropriate density of cells for microscopy. 1.5 μL of this cell suspension are mixed on a poly-lysine coated microscope slide with 1.5 μL of synthetic minimal media, containing glucose. Once released from poison re-import of NLS-GFP quickly restarts, so minimum time should lapse (less than 60 s) before the commencement of image acquisition. Time points are collected at 15 s intervals using an automated spinning disk confocal microscope (see Section 6.3). At each time point images of the NLS-GFP and the CFP-tagged subcellular markers are taken; 3 each at focal-plane separated by 1 μm increments up and down through the cells. Thus, focused images of most cells’ nuclei are acquired at each time-point, in both the NLS-GFP and Htb2-CFPp/Tpi1-CFPp fluorescence channels.
(Fig. 6A). After 10 min the NLS-GFP will have generally returned to its steady-state distribution in all cells.

6.7. Cell morphometry calculated from 3-D confocal image serial section

It is important to normalize for the different sizes and shapes of the assayed cells. Hence, a complete confocal image series should be acquired up and down through the cells, at focal-planes separated by 0.4 μm intervals (or the approximate z-axis resolution limit of the objective lens), after the last timepoints of import have been recorded (Fig. 6B). The volume of a voxel is calculated by calibrating X and Y distances with a reference slide. Thus, nuclear and cytoplasmic volumes could be measured directly from the 3-D confocal image series, by summing the number of voxels in each. The area of the nuclear envelope is calculated from a rendering of the nuclear surface, made by applying the MatLab (The Mathworks) isosurface locating algorithms to the 3-D data—similar rendering options are available in some commercial image analysis programs, such as Imaris (Bitplane AG) and Volocity (Improvision). This surface area is converted to an estimate of the number of NPCs using a figure of 12 NPCs/μm² [104], a density shown to be relatively constant throughout the yeast cell cycle. The morphological measurements taken from cells in these import assays are consistent with the values measured by Winey et al. [104]. Notably, this technique also yields accurate measurements of commercially prepared spherical fluorescent beads with known 2.5 μm diameters (Molecular Probes).

6.8. NLS-GFP cargo concentration calibrated using quantitative Western blots

The NLS-GFP concentration in a population of importing cells is calibrated using quantitative Western blotting, which compares the abundance of NLS-GFP to that of an internal standard, Htb2-CFPp (Fig. 6C). The average amount of Htb2-CFPp per cell in log-phase populations had been determined by quantitatively comparing its signal from Western blots with that of a purified
recombinant GFP standard [105], essentially as described previously [6,106]. The monoclonal anti-GFP antibody used for these experiments (Roche, Cat# 11 814 460 001) recognized GFP indistinguishable from CFP or YFP (not shown). Concentration of recombinant GFP has been determined by the Coomassie-based Bradford method [107] and amino acid analysis of triplicate hydrolysates [108]. The cell-sample and standard are mixed together and run in the same lanes to eliminate liquid handling uncertainties (150,000 cell equivalents gave a signal comparable to ~1 ng of purified GFP). Western blots are quantitated using OpenLab image analysis software (Improvision), by measuring the total pixel intensity of each band (above background) in the linear detection range. The signal from Htb2-CFPp is compared to the GFP standard calibration curve from the same blot, thus calculating an average number of copies per cell for Htb2-CFPp. The population average concentration of Htb2-CFPp, by this analysis, is 240,000 copies per cell (±18% SD); a relatively constant value between strains, making it a good choice for a concentration calibrator.

For each import assay, NLS-GFP and Htb2-CFPp bands on Western blots are quantitatively compared with each other (as above; Fig. 6C), to calculate the abundance of the cargo; for instance, a population with exactly twice the NLS-GFP signal of Htb2-CFPp would thus have an average abundance of 480,000 cargo molecules per cell. Following the assays of each day, using the same microsomal fraction, we acquire 3-D confocal image series of several fields of unperturbed cells from the same culture of cells used in the assays of that day. From these data we measure the total fluorescence of NLS-GFP in >200 of these cells, by summing the values of all voxels for each cell, which gave us a reliable measurement of the average total NLS-GFP fluorescence of our population of importing cells. Since the quantitative blotting yields the average NLS-GFP molar amount for cells in an import assay, and microscopy yields the average total fluorescence of those cells, and the total fluorescence should be proportional with total fluorophore quantity. Therefore dividing these two averages yields a calibration coefficient that can be used to convert a given cell’s fluorescence to the abundance of NLS-GFP cargo in that cell. For example, a single cell whose total NLS-GFP fluorescence was twice that of the population average would have twice the average abundance of NLS-GFP (or 960,000 copies, using the example population from before).

6.9. Analysis provides measurement of imported molecules per NPC per second for single cells

For each condition, import assays should be repeated until import data from at least ~100 cells have been collected. The CFP images of Htb2-CFPp and Tpl1-CFPp assist in segmenting each cell’s image-data into nuclear and cytoplasmic compartments, respectively, from which NLS-GFP fluorescence measurements are taken at each time point as described above.

Photobleaching can be minimized by choice of equipment and exposure conditions, but residual fluorescence loss and general signal fluctuations can also be normalized mathematically, since the total amount of NLS-GFP in a cell should be constant over the timescale of an import assay. Total cellular fluorescence is calculated at each time-point as the average fluorescence of each compartment, multiplied by that compartment’s volume and summed for all compartments. The fluorescence values of each time-point are then multiplied by the necessary factor such that the total cellular fluorescence at each time-point is maintained at a constant value. The constant value, to which the data are normalized, is the maximum total cellular fluorescence calculated at any time-point during the assay for that cell.

For each cell, the abundance of NLS-GFP therein is estimated, its distribution between the nucleus and cytoplasm at each time point is measured, and the volumes of the nucleus and cytoplasm are calculated. Therefore, calculating the import rates in these cells is a simple matter of combining these measurements and calculating the nuclear NLS-GFP concentration at each point of the time course (Fig. 6). The resulting plots of NLS-GFP concentration over time fit well to single exponential relationships (using MatLab’s Curve Fitting toolbox; average r-square of all single-cell fits should be ~0.95). Cells whose data deviate significantly from this relationship typically have either aberrant morphology, very low fluorescence signal, or suffer from excessive movement, and exclusion from the analysis is warranted. Import rates can either be calculated from the exponential fit coefficients of these curves or from a linear regression of the initial, approximately linear, time-points. Linear regression is preferred because this measure is less prone to the large fluctuations that result from slight changes in the goodness of the exponential fits. The import rates from these initial time-points are active import measurements for individual cells, free of passive diffusion, and are represented in units of cargo molecules/NPC/s.

Commercial image analysis software is typically inadequate to collect and combine so much information from so many cells in a timely manner. But, import assays can be analyzed using custom software created in MatLab (The Mathworks), developed specifically to collect, combine and analyze these import data as described above. These programs are freely available from the authors upon request, although some significant understanding of MatLab will be required for their adaptation to, and use on, a particular computer platform.

6.10. Effective import rate as a population measure of import, normalized for cargo concentration

Import rates measured for a several cells of a population are typically spread over wide ranges. This is because
the NLS-GFP expression constructs are cloned in multiple copy vectors, conferring a random number of copies of the gene to each cell with a commensurate random expression level of the fusion protein. However, this variability can also be used advantageously, by examining the relationship between cargo concentration and import-rate. The data of all cell’s import-rates and cargo concentration are split into a number of statistical bins of increasing cargo concentration, and the mean import-rate and cargo concentration within each bin can be calculated. A linear regression of these averages, fixed through the origin, typically shows a strong linear trend, the slope of which is termed effective import rate. An import efficiency value is thus a measurement of how quickly a given cargo is imported by its available transport pathway(s), in units of cargo molecules/NPCs/µM of cargo.

6.11. Tips and caveats

To obtain reliable data from these assays one needs to examine the import-rate of an NLS-GFP cargo that reached a high steady-state N/C ratio. Thus, for many such import assays, NLS-GFP fusion proteins had attached to their C-termini a single copy of the 58 amino acid Protein A (PrA) repeat sequence. This PrA fragment is not sufficient to bind antibodies in Western blots (not shown), but simply increases the cargos’ nuclear accumulation, presumably because of a decreased rate of passive leak resulting from the cargoes increased size, making the resulting data more quantifiable.

Studies have demonstrated that sodium azide and 2-deoxyglucose have many effects in the cell beyond their ability to reversibly halt nucleo-cytoplasmic transport in S. cerevisiae (Pan, X. and D.S. Goldfarb, unpublished results) [90,91]. For this reason alternative less invasive techniques have been implemented to stop transport, such as using only one of the poisons, or chilling the cells to 0 °C [90,91]. For this reason alternative less invasive methods to study these processes in even greater detail.

7. Concluding remarks

The development of new methods to study the dynamics of cellular processes continues at a fast-pace. Researchers can make directed and testable hypotheses about molecular and cellular function by combining these techniques with the wealth of genetic tools available for yeast. We have described how some of these new methods have been adapted to dissect the processes of nucleo-cytoplasmic transport in a quantitative manner in living yeast cells. As researchers, we are continually monitoring developing microscopic technologies, including such tools as photoactivatable GFP and FRAP (fluorescence recovery after photobleaching), that may provide less invasive methods to study these processes in even greater detail.

References