Selectivity of the yeast nuclear pore complex - probing transport in vivo
Popken, Petra

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Chapter 4

Towards single cell analysis of nuclear pore complex structure and function in ageing yeast

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Abstract

Ageing is a complex phenomenon and many involved molecular mechanisms are not fully understood. The nuclear pore complex (NPC) regulates nucleocytoplasmic transport in *Saccharomyces cerevisiae* and might be affected and even involved in the ageing process. *S. cerevisiae* divides asymmetrically and can be used as a model system to study replicative ageing. Here we use a microfluidics approach that allows us to follow cells throughout their lifespan. We explore strategies to quantify the abundance of NPC proteins at the nuclear envelope in single cells during ageing, and to assess the functionality of the NPC during the lifespan of a yeast cell using reporter proteins. Here we report on the advantages and disadvantages of the methods and make recommendations for future studies.

Introduction

Ageing is a complex phenomenon involving many if not all of the pathways and organelles in a cell. The question of how ageing impacts the function of the nuclear pore complex (NPC), which provides the main gateway to the nucleus in eukaryotic cells, has not been extensively addressed. One possibility is that NPC function is crucial during much of the lifetime, as life may be incompatible with a nonfunctional NPC, so large changes are not expected. At the other extreme, and also owing to it central position in eukaryotic cell biology, one may expect that altered NPC function may actually be causal to many ageing phenotypes.

The NPC is large 50 MDa assembly of ~30 different proteins called nucleoporins (nups) which are present in multiple copies. Two biochemically and functionally distinct sets of nups make up the NPC. First, a set of stably folded proteins forms a 8-fold symmetrical scaffold structure that anchors the complex to the nuclear envelope (NE) membrane. The scaffold structure is reported to be a stable assembly in postmitotic cells and its individual proteins are long lived (32, 159, 185). Second, a set of intrinsically disordered (ID) proteins is anchored to the scaffold and provides the selectivity barrier. Many of these ID proteins are mobile and move away from the NPC at times (146). In addition, in postmitotic cells these proteins were shown to continuously turnover (32). This difference in lifetimes of the various sub-complexes of the NPC can cause a loss of stoichiometry in the complex with ageing, and as a...
consequence its function may be impaired with ageing. Indeed, there is some evidence of age-related changes in NPC transport function in chronological ageing, in postmitotic cells from rats and worms. Nuclei isolated from postmitotic neuronal cells from aged rats and nuclei isolated from *Caenorhabditis elegans* were shown to be more permeable than young nuclei (32). In addition, correlations were found between the ‘leakiness’ of the nuclei and the presence of oxidative damage in the form of carbonyl modification or loss of nups at the NE.

In mitotic cells and tissues the impact of replicative ageing on NPC function and structure has been little addressed. Baker’s yeast *Saccharomyces cerevisiae* is a good model organism to study replicative ageing. An individual cell dies after ~25 divisions, the average replicative lifespan (RLS) of many yeast strains. Cells divide asymmetrically, where the daughter cell is rejuvenated while the mother cell ages with each division. This implies that the cellular contents are not divided equally, but that inheritance is regulated in a way that mother cells retain the age-related molecules. Whether NPCs are part of this collection of age-defining phenotypes depends on the question if aged-NPCs are inherited asymmetrically. This is still under debate, but it is clear that a soluble pool of the nup Nsp1 is involved in inheritance of NPCs (27, 34, 86, 111, 115, 165). Also, there is quality control in the inheritance of NPCs, as it has been shown that NPCs lacking Nup82 are selectively retained by the mother cell (115). This implies that mechanisms are in place that could control the inheritance of aged NPCs in baker’s yeast.

A recent study in baker’s yeast showed that the accumulation of several reporters with a nuclear localization signal (NLS-GFP) is lower in replicative aged cells (age 6+), showing that active transport might be affected in aged cells (106). The cellular levels of Nup116 and Nsp1 were lower in these aged cells, hinting that NPCs are affected during ageing. Our recent system-wide approach to phenotype aged cells with proteome and transcriptome data showed a more comprehensive picture of the changes in the NPC (75). Indeed, the protein level of specific nups goes down with age on a population level, while the abundances of most nups are stable (see below). Additional evidence for age-related changes in NPC biology comes from preliminary experiments showing that deletion of the FG-domains of Nup116 alone or in combination with Nup145, decreased the RLS, while cells lacking Nup100 or its FG-domain have a longer RLS (106).
Altogether there are clear indications that the NPC may be involved in replicative ageing in yeast, but a comprehensive and fully reliable picture is missing due to technical limitations. No two cells live and age identically (74) and hence single cell measurements on larger numbers of cells are important (69) to draw solid conclusions about lifespan effect of Nup deletions or age-related effects on NPC function. In addition, it remains to be seen if the observation that the cellular abundance of NPC components is unstable with ageing is related to the appearance of sub-stoichiometric NPCs at the NE, or rather that this reflects the presence of e.g. aggregates or assembly intermediates in the cytosol. Here, we present the development of new methods to map the composition and function of the NPC during ageing in single cells.

Results

Abundance of nups during ageing

First, we looked at the abundance of nups during the lifespan of a yeast cell on a population level. For this we use data from the proteome and transcriptome of ageing cells obtained by the column-captured cultivation method (75). In this method mother cells are enriched because they are captured in a column with continuous flow, which flushes out the daughter cells. Using mathematical unmixing the proteome of living mother cells is separated from the contaminating dead cells and daughter cells. Together this yields a comprehensive overview of the phenotype of ageing yeast cells over 12 time points up to 72 hours.

In Figure 4.1 the Log2 of the fold change of protein level, relative to the first time point, of different nups is plotted. When this value stays around 0, there is no change, while a value of 1 or -1 represents a 2-fold increase or decrease of the protein level, respectively. The abundance of scaffold nups, comprising linker nups (blue) and proteins from the inner ring (purple) and outer ring (red) of the NPC, is stable over 72h, whereas the inner ring nups Nup170 and Nup188 show a small increase compared to the others. The FG-nups show more deviation, with 3 nups that stand out with a more than 2-fold decrease in protein level, namely Nsp1, Nup116 and Nup2. This shows that the abundance of the different components of the NPC does not change collectively, and this might cause loss of stoichiometry in NPCs in older cells.

The population-based, whole cell levels of the abundance of nups do not give information on what happens in individual cells. Furthermore, it is
unclear if the variation in protein level in the cell means that the abundance of nups at the NPCs changes. To answer these questions we use a microfluidics approach. In the microfluidics dissection platform cells are monitored while they are trapped in a chip where the continuous flow of medium flushes out the daughter cells. In this way mother cells can be followed during their whole lifespan, without being outnumbered by the daughter cells. To visualize the nups, we constructed strains where two nups are tagged with different fluorescent proteins (GFP and mCherry). Loading these strains in the chip, cells can be followed while they are dividing and ageing, and the localization and abundance of nups can be measured using the fluorescence intensity at the NE. A change in ratio between the two fluorophores represents deviation from the stoichiometry at the NE.

Four combinations of two tagged nups were used, namely Nup100-GFP/Nup133-mCh, Nup100-GFP/Nup120-mCh, Nup133-GFP/Nup120-mCh and Nsp1-GFP/Nup133-mCh. The intensity of the two fluorescent proteins at the NE was measured immediately after loading (young) and after 21 hours (aged). Because we only have an image of 1 z-plane, we use the ratio of GFP over mCh to eliminate the effect of differences in focus because the nucleus is not in the same plane in all cells and during all times. This also corrects for

**Figure 4.1** Protein levels of NPC components in replicative ageing yeast cells. The Log2 of the fold change with respect to the first time point (7.8h) is plotted as a function of time (grey line represents a 2-fold change). Left scaffold nups: linker nups Nic96 and Nup82 in blue; inner ring nups Nup188, Nup170, Nup157 and Nup192 in purple; outer ring nups Nup84, Nup85, Nup120 and Nup133 in red. Right FG-nups (labeled) in green. (Data taken from (75))
overall change in NPC density, allowing us to compare the abundance of nups with respect to each other.

In aged cells both the GFP and mCh intensities were approximately 50% higher than in young cells, pointing to an increased density of NPCs at the NE in aged cells. Furthermore, the mCh-signal increased more than the GFP.

Figure 4.2 Microfluidics analysis of nup-abundance during ageing. A. Log2 of fold change of GFP/mCh intensity in aged cell (t = 21h) relative to young cell (t = 0h). Each point is a single cell, with the fold change plotted against the age of the cell after 21h. B. Table with the average fold change aged/young of the ratio of GFP/mCh intensity. SEM is indicated, n is number of cells analyzed.
intensity in all strains, independent of which nup was tagged, indicating that this is a systematic effect of the mCh-signal in older cells. This is most clearly seen when comparing the intensities of Nup133 tagged with GFP in yPP006 and with mCh in yPP004, where the mCh-intensities increased ~1.4 times more than the GFP-intensities. This effect could be related to GFP or mCherry specific age-dependent changes in e.g. folding kinetics or quantum yield of the fluorophores caused by changes in pH or oxidative state. We corrected for this by multiplying the mCh intensity in aged cells with a correction factor (see materials and methods). The resulting fold change of GFP/mCh ratio is plotted on a log-scale in Figure 4.2. There is a large spread in the changes, and no clear correlations can be seen between age and fold change. However, on average the abundance of FG-nups Nup100 and Nsp1 increases slightly with respect to Nup133, while the ratio between both scaffold nups Nup120 and Nup133 is more stable (Figure 4.2B). This shows that there are no big changes for the nups analyzed here, which is in line with the proteomics data for Nup100, Nup120 and Nup133, but not Nsp1. Preliminary, we conclude that after 21 hours the decline in Nsp1 levels observed on the cellular level is not observed when monitoring the levels at the NE, while stable levels of Nup120, Nup133 and Nup100 are observed in both proteome and imaging measurements. More and older cells imaged at multiple time points would be required to draw solid conclusions.

Selectivity of the NPC during ageing:

The microfluidics approach allows us to follow cells for their whole lifespan at the microscope. This also enables us to look at the selectivity of the NPC during ageing. The localization of reporter proteins can be measured at different ages in a single cell, to see if age has an effect on the steady state distribution of the GFP signal over the nucleus and cytosol, which is a reflection of leak and transport through the NPC. The reporter proteins were expressed from a genomic locus under the control of the GAL promotor, because plasmids are not maintained during replicative ageing (unpublished data G.E. Janssens, M. de Looff, L.M. Veenhoff). The reporters were expressed in a strain (JTY7) where Nup49 is tagged with mCh, which was used to indicate the position of the nucleus.

To see if active import is hampered in aged cells, we used GFP fused to tcNLS, a tandem classical nuclear localization signal. This reporter is actively imported by Kap60-Kap95 and accumulates in the nucleus. We calculated the
ratio of GFP-intensity in the nucleus over the intensity in the cytoplasm, the N/C-ratio. This ratio represents the rate of active import over passive leak. The N/C-ratio was measured when cells were just loaded in the chip (young) and after 21 hours (aged). GFP-tcNLS is stably expressed from the genomic locus and nuclear accumulation (N/C-ratio of 2.1-2.4) is maintained during ageing (Figure 4.3A). We note that cells expressing GFP-tcNLS divided more slowly than the strains expressing tagged nups, as after 21h we obtained an average age ~8 when expressing GFP-tcNLS (Figure 4.3A) versus an average age ~13 with the strains expressing tagged nups (Figure 4.2A). This may be related to the expression of GFP-tcNLS but likely also reflects the different carbon source: raffinose instead of glucose causes a lower growth rate.

Overall, we conclude that reporter proteins can be expressed from a genomic locus under control of the GAL promoter, which allows for analysis of the localization of the reporter during the lifespan of the mother cell. However, the different carbon source that is used for these experiments causes slower growth, so to obtain data for older cells, longer experiments should be performed. In addition, as we observed large cell to cell variation, 50-100 of cells should be followed over at least 5 time points over the full life span to obtain a complete data set.

Next to active import we wanted to determine if active export might be affected in older cells. For this we fused GFP to the nuclear export signal

![Figure 4.3](image)

**Figure 4.3** Microfluidics analysis of GFP-tcNLS localization during ageing.  
A. Log2 of fold change of the N/C ratio in aged cell (t = 21h) relative to young cell (t = 0h). Each point is a single cell, with the fold change plotted against the age of the cell after 21h.  
B. Table with the average N/C ratio in young and aged cells and the average fold change (aged/young). SEM is indicated, n is number of cells analyzed, total from 2 chips.
(NES) from Ssb1. Expression of GFP-NES (induction with galactose) resulted in slowly dividing, sick cells. Therefore, we used the low level expression observed in un-induced cells grown on raffinose to determine the localization of this reporter. Even with this low expression level cells with GFP-NES still divide slowly. After 60h the average age is ~8, while GFP-tcNLS cells already have an age of 8 after 21h (compare Figure 4.4 and Figure 4.3A). It is unclear what causes the slow division, but it may well be related to competition with native active export for Crm1, the export factor.

To improve the throughput of the method, we used the ALCATRAS chip (31), which allowed us to follow more cells in one experiment. The N/C ratio was measured at multiple time points after 0, 15, 30, 45 and 60 hours. After the last time point, the cells were followed until they died to obtain the full lifespan of all cells. This way we can also observe when changes occur in cells, not only at a certain age (number of divisions) but also at a percentage of total lifespan. With this improved set up, using multiple time points and increased throughput, we can follow the transport efficiency of individual cells throughout their lifespan, and it shows the potential of this method.

Discussion

Ageing is a complex phenomenon, and the molecular mechanisms that are involved are not yet understood. A recent proteomics and transcriptomics study in yeast provided us with proteome and transcriptome data over the complete lifespan (75). This gave information towards the global signatures of ageing, but it also provided more detailed data on specific cellular components.

![Figure 4.4](image_url) Example of results from microfluidics method using ALCATRAS chip and multiple time points, using GFP-NES localization. Log2 of fold change of the N/C ratio at 4 time points (t = 15, 30, 45 & 60h), relative to young cell (t = 0h) is plotted. Each line represents a single cell in which the fold change is plotted against the age of the cell.
Here we looked at the behavior and role of the NPC in ageing cells. The proteome data from Janssens et al showed that the abundance of most scaffold nups does not change in mother cells, while the levels of some FG-nups are more variable (75). Three FG-nups, Nsp1, Nup116 and Nup2, decrease more than 2-fold in old cells compared to the first time points.

We set up a method using a microfluidics approach that can be used to confirm these changes on a single cell level, and to see if the changes represent the abundance of the proteins in the NPC. We tagged nups with two fluorescent proteins (GFP and mCh) and measured the ratio between the two intensities. In this way we could obtain the relative abundance of the different nups at the NE. We noticed that GFP and mCh showed different behavior in aged cells, because the mCh intensity increased more compared to the GFP signal when the reporter was fused to the same nup. It is possible that the difference is due to a difference in maturation of the fluorescent proteins, or the proteins are differently affected by the biochemical/biophysical environment of aged cells. Provisionally we corrected for these, by calculating a correction factor using different strains where the same nup was tagged with GFP or mCh (see Materials & Methods). The correction factor is imprecise because the behavior of the fluorescent proteins might be affected by the location in the NPC. Furthermore, because an average over only a few cells with a wide variation in ages was used, real biological changes in individual cells may have affected the correction factor. Corrections are better done in the future by precisely mapping out the effects of ageing on the fluorophores by creating strains where the tags are swapped.

Using the microfluidic chips, we obtained data on the relative abundance of Nup100, Nsp1, Nup133 and Nup120 at the NE. The scaffold nups Nup133 and Nup120 show similar behavior and do not change with respect to each other, as was also seen in the cell-based proteome analysis. FG-nups Nup100 and Nsp1 increase slightly compared to the scaffold nups. This does not correspond to the decrease in Nsp1 observed in the proteome data, which started around ~20h with a further decline after ~60h (75), and in immunoblotting data of lysates of age 6-9 cells reported in (106). This could mean that the measurements may have been performed at a too early (young) time point, i.e. that the levels of Nsp1 are not yet reduced. Alternatively, the decrease in Nsp1 observed in the proteome study may be caused by a decline in the cytoplasmic pool of Nsp1, which is involved in inheritance of NPCs.
(27, 115). Thus, the decrease in the soluble pool might have consequences for the transmission of NPCs towards the NE of daughters of old mothers.

Unfortunately, there is no data yet on Nup116 and Nup2. It would be interesting to see if the decreased proteome levels reflect the abundance levels of these nups at the NE. Especially for Nup116 it will be important to address the levels in the NPCs, as Nup116 is known to be critical for maintaining the permeability barrier (106), next to Nup100 (144). In addition, it would be valuable to follow cells for their complete lifespan and measure the abundances at multiple time points, as was done for cells with the GFP-NES reporter. This way we can get more robust data sets that are less sensitive to measurement errors, and determine on a single cell level if changes in the NPC stoichiometry might correlate with lifespan or cell death.

The microfluidics approach can also be applied to see if the transport properties of the NPC are changed in aged cells. We measured the localization of GFP-reporters with a NLS or NES. The N/C ratios measured are not a direct report on transport efficiency, as passive diffusion also impacts the localization of both reporters. When the transport machinery has to pump against a larger leak through the NPC, the accumulation will decrease. So to get a complete picture, also a reporter for passive diffusion should be included in the study. Expression of GFP-NES dramatically decreased the lifespan of cells, and even at low expression, when cells are grown on raffinose, already slowed down cell division and RLS. Possibly the (over)expression of GFP-NES is causing a too big load on the export machinery, specifically on transport factor Crm1 that is responsible for export of NES-proteins. Crm1 (Xpo1) is an important export factor, as it is also involved in the export of mRNA. A knock-out is lethal, and a growth defect is also seen in cells with a mutant xpo1-1 (173), so the expression of GFP-NES might have the same effect as the mutant. A possibility to relieve these potentially negative effects from competition with native export may be to work with a GFP-NES variant encoding a lower affinity NES signal.

In conclusion: we present a new approach to study the changes in NPC stoichiometry and selectivity during ageing in single cells. This approach has great benefits over previously used methods (106) as it allows longitudinal studies of large numbers of cells, which is critical considering the large cell to cell variation during ageing. Future studies should be aimed at generating a larger dataset over many cells and timepoints to further improve the analysis.
Materials & Methods

Strains, plasmids & growth conditions

Strains used in this study are all in the BY4741 background, the specific strains are listed in Table 4.1. Plasmids (Table 4.2) were generated by standard molecular biology techniques and validated by sequencing; details are available upon request. Double tagged-nup strains yPP004-yPP007 were constructed by homologous recombination. Primers with specific overhangs for each nup were used to amplify the mCh-tagging cassette from pPP014 and the PCR product was used to transform a nup-GFP strain from the GFP-collection (71). Strains yPP008-yPP011 with genes for reporter proteins integrated in the genome were made using integrative plasmids pPP042-pPP044 and pPP046, respectively. Plasmids pPP042-pPP044 and pPP0046 are based on pRS303 (169) and contain Gal1-promoter, reporter gene and terminator, cloned using SacI and EagI from related pUG-plasmids. The integrative plasmids were linearized using NsiI before transformation of yeast strain JTY7 (Nup49-mCh).

For experiments with double tagged-nup strains, cells were grown at 30°C in a flask with selective drop-out medium, supplemented with 2% glucose to obtain an exponentially growing culture. For experiments with reporter strains, cells were grown at 30°C in a flask with selective drop-out medium, supplemented with 2% raffinose to obtain an exponentially growing culture. Reporter protein GFP-teNLS was expressed under control of the GAL1 promoter by induction with 0.5% D-galactose for ~6h before loading in the chip. Expression of GFP-NES resulted in slowly dividing, sick cells, so for experiments with this reporter the low expression of uninduced cells grown on raffinose was used to analyze the localization.

Microfluidics Dissection Platform & Microscopy

Exponentially growing cells were loaded in the microfluidics dissection platform (70, 101) or in the ALCATRAS chip (31) at the DeltaVision Microscope. Experiments were run for the indicated hours inside a climate chamber at 30°C with continuous flow of fresh medium. Imaging was done on a DeltaVision Deconvolution Microscope (Applied Precision), using InsightSSITM Solid State Illumination of 488 and 594 nm and an Olympus UPLS Apo 100x oil objective with 1.4NA. Detection was done with a CoolSNAP HQ2 camera. Data was analyzed with open source software Fiji (160).
Calculating ratio change of GFP/mCh in aged and young cells

To calculate the change in ratio of GFP/mCh in the double-tagged nup strains, we need a correction factor, because in all strains the mCh-intensity increases more than the GFP-intensity, independent of which nups are tagged. Because we have the three combinations Nup100-GFP/Nup133-mCh (yPP004), Nup100-GFP/Nup120-mCh (yPP005) and Nup133-GFP/Nup120-mCh (yPP006), we can compute a correction factor that takes into account the relative age-related increase in intensity of mCherry, as follows.

In the equations below the calculations for the mean ratios are written, with equation 1: yPP004 x yPP006 = yPP005.

Table 4.1 *S. cerevisiae* strains used in this study

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<th>Genotype</th>
<th>Source</th>
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<tr>
<td>Nup100-GFP</td>
<td></td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nup100-GFP::HIS</td>
<td>(71)</td>
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<tr>
<td>Nup133-GFP</td>
<td></td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nup133-GFP::HIS</td>
<td>(71)</td>
</tr>
<tr>
<td>Nsp1-GFP</td>
<td></td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Nsp1-GFP::HIS</td>
<td>(71)</td>
</tr>
<tr>
<td>yPP004</td>
<td>Nup100-GFP Nup133-mCh</td>
<td>Nup100-GFP Nup133-mCh::URA</td>
<td>This study</td>
</tr>
<tr>
<td>yPP005</td>
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<td>Nup100-GFP Nup120-mCh::URA</td>
<td>This study</td>
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<td>yPP006</td>
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<td>Nup133-GFP Nup120-mCh::URA</td>
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<tr>
<td>yPP007</td>
<td>Nsp1-GFP Nup133-mCh</td>
<td>Nsp1-GFP Nup133-mCh::URA</td>
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<td>JTY7</td>
<td>Nup49-mCh</td>
<td>MATα NUP49-mCherry::CaURA3, can1Δ::STE2pr-LEU2, ura3Δ0, lyp1Δ, leu2Δ0, his3Δ1, met15Δ0</td>
<td>(184)</td>
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<td>Nup49-mCh::URA GFP-NES(Galpromoter)::HIS</td>
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By eliminating the Nup133-terms, we obtain equation 2. From this we calculated the correction factor $c = 0.71$ for mCh-tagged nups in aged cells.

\[
\frac{Nup100\text{aged}/(c \times Nup133\text{aged})}{Nup100\text{young}/Nup133\text{young}} \times \frac{Nup133\text{aged}/(c \times Nup120\text{aged})}{Nup133\text{young}/Nup120\text{young}} = \frac{Nup100\text{aged}/(c \times Nup120\text{aged})}{Nup100\text{young}/Nup120\text{young}}
\]

\(1\)

\[
\frac{Nup100\text{aged}}{Nup100\text{young}} \times \frac{1/Nup120\text{aged}}{1/Nup120\text{young}} = c \times \frac{Nup100\text{aged}/Nup120\text{aged}}{Nup100\text{young}/Nup120\text{young}}
\]

\(2\)

### Table 4.2 Plasmids used in this study

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<td>pAC06 GFP</td>
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